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Changing the functionality of cocoa butter

Thesis submitted in fulfillment of the requirements for the degree of
Doctor (PhD) in Applied Biological Sciences: Chemistry

I did it my way …

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Samenvatting

Chocolade is een suspensie van deeltjes (suiker, cacao en eventueel melkpoeder) in een continue fase van cacaoboter. Cacaoboter is een essentieel ingrediënt aangezien het verantwoordelijk is voor de glans, de textuur en het typische smeltgedrag van 'onweerstaanbare' chocolade. In dit onderzoek werd de functionaliteit van cacaoboter op twee manieren gewijzigd. In het eerste deel werd de rauwe cacaoboter geraffineerd en de invloed van dit proces op de uiteindelijke kwaliteit van melkchocolade werd onderzocht. In het tweede deel werd cacaoboter enzymatisch gemodificeerd via een glycerolyse reactie. Op die manier werden cacaoboter diglyceriden geproduceerd. Deze werden chemisch gekarakteriseerd, hun kristallisatiegedrag werd onderzocht en in een laatste deel werd ook hun functionaliteit in chocolade geëvalueerd.

Hoofdstuk 1 start met achtergrondinformatie over de herkomst van cacaoboter. De karakteristieke chemische samenstelling, die verantwoordelijk is voor zijn unieke fysische eigenschappen, werd verder in detail beschreven om te eindigen met een beschrijving van de verschillende stappen van het productieproces van chocolade.

Cacaoboter moet vanuit een sensorisch (smaak, geur, kleur) en technologisch standpunt vrij zijn van ongewenste smaakcomponenten en ranzigheid en in overeenstemming met de EU-richtlijn 2000/36/EG (2000) minder dan 1.75% vrije vetzuren bevatten. Daarnaast gebruiken fabrikanten vaak ook verschillende cacaoboters naargelang de gewenste kleur en smaak. Door de toenemende vraag naar chocoladeproducten daalt daarenboven de kwaliteit van de cacaoboter. Omwille van deze factoren is er vaak een intens raffinageproces nodig om een kwalitatieve cacaoboter te bekomen. Studies over de impact van een raffinageproces op de uiteindelijke chocoladekwaliteit zijn echter beperkt. Daarom werden er in de studie in Hoofdstuk 2, drie ruwe cacaoboters, met of zonder een silica voorbehandeling, onderworpen aan een stoomraffinage in een gepakte kolom bij vijf temperaturen: 150°C, 175°C, 200°C, 225°C en 250°C. De geraffineerde cacaoboters werden daarna gebruikt voor de productie van melkchocolades.
Het belangrijkste effect van de silica voorbehandeling was de volledige verwijdering van fosfor (dus fosfolipiden), ijzer en alkalische componenten. Verder werden tijdens de stoomraffinage de vrije vetzuren bij een temperatuur hoger of gelijk aan 200°C bijna volledig verwijderd. De geobserveerde veranderingen in de fysische eigenschappen waren

voornamelijk gerelateerd met de verwijdering van deze vrije vetzuren. De vrije vetzuren zorgden voor een latere en tragere kristallisatie en naast het effect op de kristallisatiekinetiek werd ook een lager vast vetgehalte waargenomen.
De temperatuur van de stoomraffinage had voornamelijk een invloed op het vloeigedrag van de melkchocolades. Het verwijderen van de vrije vetzuren resulteerde in een lagere zwichtspanning en een hogere viscositeit. Het was duidelijk dat de vrije vetzuren een belangrijke rol speelden maar hun mechanisme bleef onduidelijk.

In het tweede deel van dit onderzoek werd de chemische samenstelling van cacaoboter meer drastisch gewijzigd door een enzymatische glycerolyse reactie, met als hoofddoel de productie van cacaoboter gebaseerde diglyceriden.
Door de aanwezigheid van een vrije hydroxylgroep hebben diglyceriden andere fysisco-chemische eigenschappen dan de corresponderende triglyceriden. Bovendien blijken voornamelijk 1,3-diglyceriden nutritionele voordelen te hebben in vergelijking met triglyceriden.

In Hoofdstuk 3 werd eerst gezocht naar een zo eenvoudig mogelijk productieproces voor de diglyceriden zonder solventgebruik. Op basis van literatuuronderzoek werd gekozen voor een enzymatische glycerolyse reactie. De grenzen van de verschillende reactieparameters werden vastgelegd en daarna werd de reactie geoptimaliseerd met response surface methodology. Onder optimale reactie omstandigheden was het mogelijk om 50% diglyceriden te verkrijgen. In de laatste stap van het productieproces werden de cacaoboter diglyceriden opgezuiverd via een short-path destillatie. Hierbij werden de beste druk-temperatuur combinaties bepaald voor de vier verschillende stappen van het destillatieproces. Het was mogelijk om in de laatste destillatie stap een zuivere diglyceridenfractie te verkrijgen bij een zeer laag vacuüm en een temperatuur van 260°C en dit met een procesrendement van 93%.

De fysico-chemische eigenschappen van de geproduceerde cacaoboter diglyceriden werden in Hoofdstuk 4 gekarakteriseerd. Ze bevatten ongeveer 50% mono-onverzadigde, 37% di-verzadigde en 11% di-onverzadigde diglyceriden. Door co-kristallisatie en onderlinge oplosbaarheid werden er drie endotherme pieken waargenomen in het smeltprofiel. De diglyceriden werden daarna gemengd met cacaoboter om hun toepasbaarheid te screenen. De niet-isotherme kristallisatie en smelteigenschappen werden bestudeerd met behulp van differentiële scanning calorimetrie (DSC), terwijl gepulste nucleaire magnetische resonantie (pNMR) werd gebruikt om het gehalte aan vast vet als functie van de temperatuur te

bepalen. De invloed van de verschillende diglycerideconcentraties op de microstructuur werd gevisualiseerd door gepolariseerde lichtmicroscopie (PLM). De kristallisatie onset nam evenredig toe met de hoeveelheid diglyceriden, voornamelijk door aanwezigheid van een hoogsmeltende fractie. Het karakteristieke smelt- en kristallisatiegedrag van cacaoboter werd geleidelijk veranderd door toevoeging van diglyceriden. Het isosolid diagram, karakteristiek voor het fasegedrag van de mengsels, vertoonde een eutectisch minimum, wijzend op een verzachtend effect van de diglyceriden. Een groot nadeel was de hoge hoeveelheid aan resterend vast vet bij temperaturen boven de lichaamstemperatuur wat bij toepassing kan resulteren in een ongewenst wasachtig mondgevoel. Uit de visualisatie van de microstructuur bleek dat de morfologie van het kristalnetwerk sterk wijzigde bij toevoeging van diglyceriden.

In Hoofdstuk 5 werd een multi-methodologische benadering gebruikt om de isotherme kristallisatie van cacaoboter bij 20°C, in de aanwezigheid van maximaal 10% cacaoboter diglyceriden, over een periode van vier uur te bestuderen. DSC en pNMR werden toegepast om inzicht te krijgen in de primaire kristallisatie, terwijl oscillatorische reologie informatie verstrekt over zowel de primaire kristallisatie als de microstructurele ontwikkeling. X-stralen diffractie werd gebruikt om de polymorfe vorm en kristalpakking te bepalen. De microstructuur werd opnieuw gevisualiseerd door PLM.
Bij de geselecteerde isotherme kristallisatietemperatuur van 20°C kristalliseert cacaoboter in twee stappen: de α kristallen gevormd tijdens de eerste stap worden omgezet tot β’ in de tweede kristallisatiestap. De hoogsmeltende di-verzadigde diglyceriden wijzigen de verzadigingscondities van de smelt en ze treden op als kernen voor de verdere kristallisatie. De inductietijd van de eerste kristallisatiestap was omgekeerd evenredig aan de hoeveelheid diglyceriden. De daaropvolgende polymorfe transitie werd vertraagd door de aanwezigheid van de diglyceriden daar ze de hexagonale packing stabiliseren. De gelijkaardige vetzuursamenstelling van de diglyceriden en de cacaoboter heeft waarschijnlijk bijgedragen tot dit effect. Tot 2.5% diglyceriden kunnen ook β' kristallen uit de smelt gevormd worden in de tweede kristallisatiestap. Bij hogere concentraties gebeurt deze transformatie enkel via de α-vorm. Bij 5% of meer diglyceriden werd op het einde van de isotherme periode reeds een diffractiepiek teruggevonden die karakteristiek is voor β_V. Het lijkt dan ook interessant om in toekomstig onderzoek een langere isotherme kristallisatieperiode te onderzoeken.

In het laatste hoofdstuk, Hoofdstuk 6, werd in de receptuur van een donkere chocolade tot maximum 25% van de cacaoboter vervangen door diglyceriden. De functionele eigenschappen werden bepaald door het smelt- en vloeigedrag en de textuur. Tot 5%

diglyceriden waren de verschillen met de referentiechocolade klein. Hogere concentraties zorgden voor een lager piekmaximum en een hogere Casson zwichtspanning.

Aangezien migratievetbloem nog steeds een belangrijk probleem is voor veel chocoladeproducenten, werd onderzocht of de diglyceriden dit verschijnsel konden voorkomen. Hiervoor werden modelsystemen gebruikt bestaande uit een cilinder gevuld met chocolade en/of vulling. De modelsystemen zonder vulling deden dienst als referentie. Het oppervlak van de chocolade werd op regelmatige tijdstippen onderzocht om vetbloemkristallen op te sporen en beoordeeld door een geautomatiseerde beeldanalyse procedure. Daarna werden de chocoladelaagjes versneden in verschillende lagen en verder geanalyseerd met HPLC en DSC om de oliemigratie in kaart te brengen.

De chocolades zonder vullingen vertoonden nauwelijks wijzigingen gedurende de opslagperiode van een jaar. Wat betreft de chocolades in contact met de vulling toonden DSC en SEM ontegensprekelijk aan dat de oliemigratie de activerende factor was voor omkristallisatie tot β_{IV} kristallen die waargenomen worden als vetbloem. Het model dat de oliemigratie beschrijft op basis van Fickiaanse diffusie met een constante diffusiviteit is niet nauwkeurig genoeg. Niet-Fickiaans gedrag trad op aangezien naast migratie de interactie tussen de olie en de cacaoboter een belangrijke rol speelt. De aanwezige diglyceriden vertraagden, noch voorkwamen het optreden van vetbloem.

Summary

Cocoa butter is an essential ingredient in chocolate as it forms the continuous phase of chocolate. It's therefore responsible for the gloss, texture and typical melting behaviour of 'irresistible' chocolate. The aim of this research was to change the functionality of cocoa butter by two different methods. In the first part, cocoa butter was modified by physical refining and the influence on final milk chocolate quality was investigated. In the second part, cocoa butter was subjected to an enzymatic glycerolysis process to produce cocoa butter diacylglycerols. These were characterized and the crystallization behaviour in combination with cocoa butter was investigated. In the last part, the functionality of the cocoa butter based diacylglycerols in chocolate was evaluated.

Chapter 1 starts with giving some background information about the origin of cocoa butter. The characteristic chemical composition, responsible for its unique physical properties, is further described in detail. The different steps of the chocolate production process are further explained as cocoa butter is mainly used to produce chocolate.

Cocoa butter has to contain less than 1.75% free fatty acids (FFA, based on oleic acid) to be in compliance with the EU directive 2000/36/EC (2000) and needs to be free from off-flavours and rancidity from a sensory (taste, odour, colour) and technological point of view. Due to an increasing demand for chocolate products, the quality of the cocoa butter tends to decrease. Additionally, manufacturers also require different types of cocoa butters (e.g. in terms of colour, degree of neutral flavours). Consequently, the application of a more intense refining process is necessary to obtain a good quality cocoa butter. However, information about the impact of different refining conditions on final chocolate quality is limited. Therefore, in the study of Chapter 2, three crude cocoa butters with or without preceding silica pretreatment, were subjected to a steam refining in a packed column at five temperatures: 150°C, 175°C, 200°C, 225°C and 250°C. In this way it was possible to produce milk chocolates containing cocoa butter with a different refining history.
The major effect of the silica pretreatment was the complete removal of phosphorous (thus phospholipids), iron and alkaline components. Furthermore, at increased temperature ($T \geq$ 200°C), the free fatty acids were almost completely eliminated by the steam-refining. The changes in the physical properties of cocoa butter were mainly related to the removal of these fatty acids. The free fatty acids retarded and slowed down the crystallization and

besides the effect on crystallization kinetics, a lower solid fat content was obtained at equilibrium conditions.

The major outcome of this study was that the temperature during packed column steam refining had an impact on rheological properties of the milk chocolate. The removal of the free fatty acids resulted in a lower yield stress and a higher viscosity. It was clear that the free fatty acids played a predominant role but the exact mechanism remains unclear.
From this study it could be concluded that by tuning the refining conditions (pretreatment or no pretreatment, temperature of the refining process), the cocoa butter properties can be manipulated to suit the desired chocolate properties.

In the second part of this research, cocoa butter was more drastically changed as it was submitted to an enzymatic modification process, aiming to produce diacylglycerols. Diacylglycerols have distinct physicochemical properties compared to triacylglycerols due to the presence of a free hydroxyl group. Moreover, nutritional studies have revealed that diacylglycerols, in which 1,3-diacylglycerols are the major components, may have nutritional benefits in comparison with triacylglycerols.

In Chapter 3, the first step was to define an appropriate synthesis pathway, avoiding the use of solvents and keeping the reaction as simple as possible. Therefore, enzymatic glycerolysis was the method of choice. The different reaction parameters within fixed boundaries were determined, to define the optimal conditions with response surface methodology. At the optimal reaction conditions, it was possible to obtain 50% diacylglycerols. The last step of the production process was to define the most suitable pressure-temperature combinations for the four-step short path distillation process. In the last distillation step, it was possible to obtain a highly purified diacylglycerol fraction at a very low vacuum and a temperature of 260°C, with an acceptable process efficiency of 93%.

In the next part of the research (Chapter 4), the physicochemical properties of the produced cocoa butter diacylglycerols were characterized. They contained around 50% monounsaturated, 37% disaturated and 11% diunsaturated diacylglycerols. Co-crystallization and intersolubility of these diacylglycerols resulted in three main melting areas. The diacylglycerols were blended with cocoa butter to screen their applicability in confectionary products. The non-isothermal crystallization and melting behaviour was studied by differential scanning calorimetry (DSC) while pulsed nuclear magnetic resonance (pNMR) was used to determine the solid fat content as function of temperature. The influence of the different diacylglycerol concentrations on the microstructure was visualized

by polarized light microscopy (PLM). It was observed that the onset of crystallization increased with an increasing amount of diacylglycerols due to the presence of the high-melting fraction. The characteristic melting/crystallization peak of cocoa butter, gradually changed upon addition of diacylglycerols. In the isosolid diagram, illustrating the phase behaviour of the blends, the isosolid lines showed distinct an eutectic minimum indicating a softening effect of the diacylglycerols. A major disadvantage was their high amount of residual solid fat above body temperature resulting in an undesired waxy mouth feel, therefore limiting their applicability. Visualization of the microstructure revealed that morphology of the fat crystal network was significantly changed when diacylglycerols were added.

In Chapter 5, a multi-methodological approach was used to study the isothermal crystallization of cocoa butter, in the presence of maximum 10% cocoa butter diacylglycerols over a period of four hours. DSC and pNMR were applied to gain insight in the primary crystallization, while oscillatory rheology provided information on both primary crystallization and microstructural crystal network development and strength. X-ray diffraction was used to determine undoubtedly the polymorphic form and crystal packing. Furthermore, the microstructure was again visualized by PLM.
It is known that at the selected isothermal crystallization temperature of 20°C, cocoa butter crystallizes in a two-step process: the α crystals, formed during the first step, transform to β' in the second step. The high-melting disaturated diacylglycerols changed the saturation conditions of the melt and acted as seed crystals. The induction time of the first crystallization step was therefore inversely related to the amount of diacylglycerols. The subsequent polymorphic transition on the other hand, was delayed by the presence of the diacylglycerols as they seem to stabilize the hexagonal packing. The good match in fatty acid composition between the diacylglycerols and cocoa butter may have contributed to this effect. Up to 2.5% diacylglycerols, besides solid-solid transition, β' crystals were also directly formed from the melt. At higher concentration, the β' formation was only α-mediated. In future research it can be interesting to study the polymorphic behaviour over a longer storage period as when 5% or more diacylglycerols were present, the XRD diffraction patterns already showed a typical peak of the β_V.

Finally, in Chapter 6, the cocoa butter based diacylglycerols were applied in a dark chocolate formulation up to 25% on fat base and the functional properties were derived by defining the melting and flow behaviour and texture. Up to 5% diacylglycerols the physical characteristics of the chocolates were not significantly influenced compared to the reference chocolate.

Higher amounts of diacylglycerols slightly decreased the peak maximum while the Casson yield stress was significantly higher.

As migration fat bloom remains one of the major quality issues in composed chocolate products, it was investigated whether cocoa butter based diacylglycerols could delay or prevent this phenomenon. A model system consisting of a cylinder filled with filling and chocolate was utilized for this purpose. Chocolates without filling were used as control samples. The surface of the chocolate was examined at regular time intervals to detect the presence of fat bloom crystals and assessed by an automated image analysis procedure. The chocolate was cut into different layers and analyzed by HPLC and DSC to monitor the oil migration. It was noticed that the quality characteristics of the plain chocolates only slightly changed during the one year storage period. In the composed chocolate products, DSC and SEM demonstrated that the oil migration was a triggering factor for the re-crystallization to β_{VI} crystals, protruding through the chocolate surface resulting in a greyer and duller appearance. The model based on Fickian diffusion with a constant diffusivity failed to accurately describe the migration of oil into chocolate. Non-Fickian behaviour occurs as besides migration, interaction between oil and cocoa butter can also play a major role. The present diacylglycerols did not delay oil migration, nor prevented the appearance of migration fat bloom.

Outline of the research

Cocoa butter is one of the main ingredients of chocolate. Due to its unique TAG composition it's mainly responsible for the gloss, texture and typical melting behaviour of chocolate. In this research two tools were used to modify cocoa butter as presented in Figure 1.

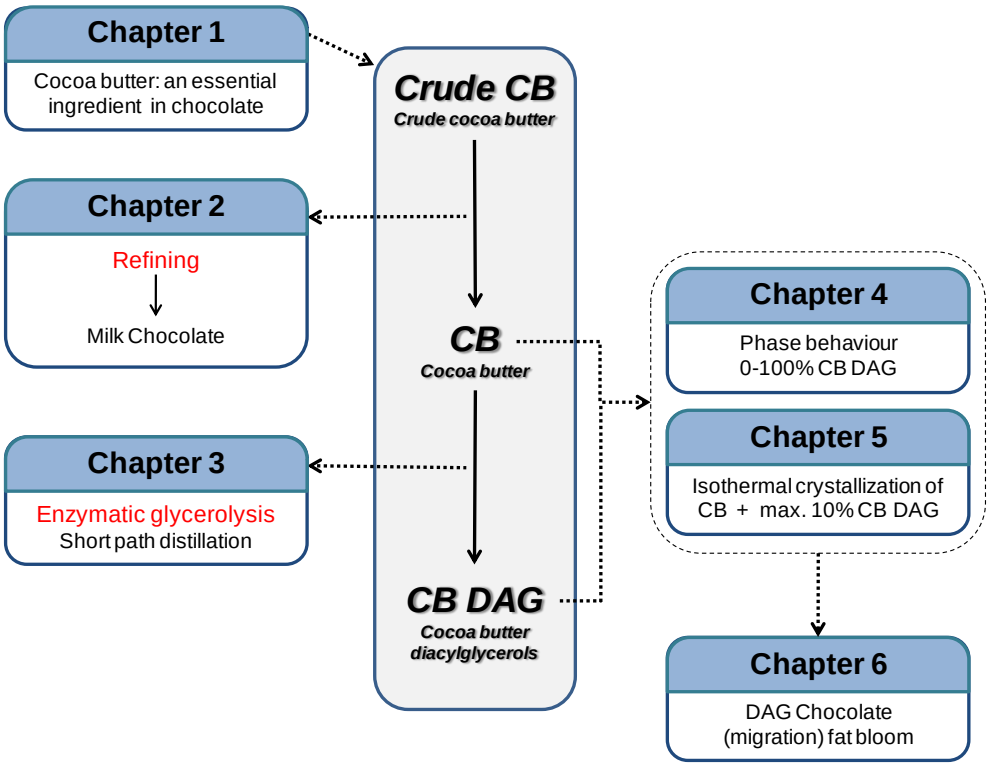


Figure 1 Outline of the research

The first chapter gives some background information about the origin of cocoa butter. The physicochemical properties are described, to end with an overview of the chocolate production process.

In Chapter 2, the physicochemical properties and thus the functionality of cocoa butter was changed by physical refining. Different refining conditions were applied and the impact on the physicochemical properties of cocoa butter and the subsequent use in milk chocolate was evaluated by different techniques.

In the second part of the research, cocoa butter was more drastically changed by enzymatic modification. The cocoa butter was subjected to an enzymatic glycerolysis process aiming at

the production of cocoa butter based diacylglycerols (CB DAG). Chapter 3 describes the development of the enzymatic reaction and the purification by short path distillation.

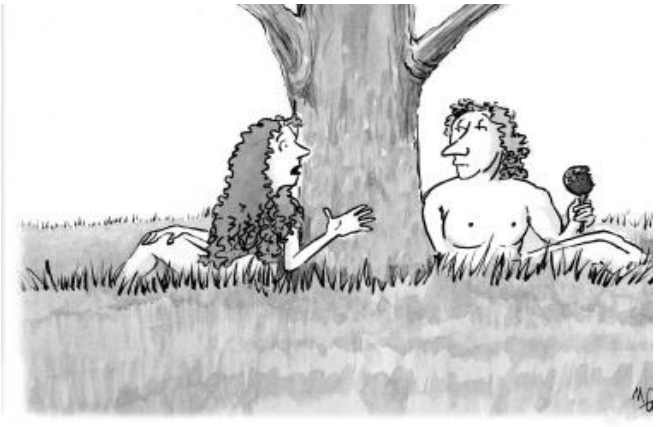
In Chapter 4, the produced cocoa butter based diacylglycerols were characterized. They were blended in different ratios with cocoa butter and the phase behaviour was investigated to screen their possible applicability in confectionary products.

Essential sensory and functional characteristics in fat based products are determined by different levels of structure within the crystal network, which are formed by their constituent lipid species. For that reason, a more fundamental study was performed on the isothermal crystallization behaviour of coca butter in the presence of up to 10% cocoa butter based diacylglycerols. The results of this study are described in Chapter 5.

Finally, in Chapter 6, the cocoa butter based diacylglycerols were applied in a dark chocolate formulation and the functional properties were derived. As migration fat bloom remains one of the major quality issues in composed chocolate products, it was investigated whether cocoa butter based diacylglycerols could delay or prevent this phenomenon.

Chapter 1

Cocoa butter: an essential ingredient in chocolate



There are extenuating circumstances when you consider that the apple was covered in dark chocolate and rolled in macadamia nuts

1.1 Introduction

Cocoa butter is an essential ingredient of chocolate as it forms the continuous phase of chocolate and it is responsible for the gloss, texture and typical melting behaviour of 'irresistible' chocolate. This chapter starts with some historical background and the transformation of the cocoa pod into the major chocolate ingredients are described. This is followed by a more detailed discussion of the physicochemical properties of cocoa butter. The last section deals with the application of cocoa butter in the chocolate production process.

1.2 Origins of cocoa and its spread around the world

The genus *Theobroma* is a genus of flowering plants in the mallow family, *Mavaceae*. *Theobroma* is divided into 22 species of which *Theobroma cacao*, the cocoa tree, is the most widely known. The generic name is derived from the Greek words Theos and Bromos, meaning God and oats respectively, so it can be called "food of the Gods". It were the Maya who have provided tangible evidence of cacao as a domesticated crop. Archaeological evidence in Costa Rica indicates that cacao was drunk by Maya traders as early as 400 BC. The Aztec culture placed much emphasis on the sanctity of cacao. The cocoa beans served as means of exchange and were used in a cocoa drink, Xocoatl. It was Hernan Cortés, leader of an expedition in 1519 to the Aztec empire, who returned to Spain in 1528 bearing the Aztec recipe for xocoatl with him. The drink was initially received unenthusiastically and it was until sugar was added that it became a popular drink in the Spanish courts and the demand over whole Europe began to increase during 17^{th} and 18^{th} century. At this point in history, chocolate was still consumed in liquid form and was mainly sold as pressed blocks of a grainy mass to be dissolved in water or milk to form a foamy chocolate drink.

It was not until 1828 that the production of cocoa and chocolate was truly revolutionized by the invention by Coenraad Van Houten of a cocoa press which succeeded in separating cocoa solids from cocoa butter. The defatted cocoa powder was much easier to dissolve in liquid and paved the way in 1848, for the invention of the first real "eating chocolate", produced form the addition of cocoa butter, sugar and cocoa mass (Dand, 1999; Dhoedt, 2009).

1.3 Growing cocoa

Cocoa is produced in countries within 20°N and 20°S of the Equator where the climate is appropriate for growing cocoa trees. It is a small, evergreen tree of 12-15 m height. Flowers

and hence the fruits (cocoa pods) grow from the trunk and thicker branches. There are three main growing areas: West Africa, South East Asia and South America. The largest producing countries are Ivory Coast (39%), Ghana (21%) and Indonesia (13%). The production is highly concentrated in West Africa (around 70%) which means that the supply is vulnerable to a number of factors such as the spread of pests and diseases, climatic conditions and political stability. Cocoa is also facing competition from other crops such as oil palm, Robusta coffee, rubber and cloves. About 90% is grown by smallholders usually on farms with a mixed cropping system (Fowler, 2009).

Apart from being choosy as regards location, soil conditions and climate are important in encouraging optimum growth. The soil has to be fairly loose and the climate humid. The rainfall should range preferably between 1500 and 2500 mm, evenly distributed throughout the year, temperature should be between 18 and 30°C and cocoa trees must not receive too much sun, nor too much shade. Therefore the natural habitat of the cocoa tree is in the lower storey of the evergreen rainforest.

Two sub-species of *Theobroma cacao* have been identified and are classed as *Theobroma cacao ssp cacao* and *Theobroma cacao ssp sphaerocarpum* (Forastero). The first may be considered as the *Criollo* cocoa and the latter are mainly referred to as *Amelonado* and *Trinitario*. *Criollo*, the cocoa tree used by the Mayas, have white cotyledons and a mild, nutty cocoa flavour. They are susceptible to diseases and produce low yield so they are highly prized and rare. The second type, *Forastero*, originate from Bahia in Brazil and include several sub-varieties. The *Amelonado* type cocoa is probably the largest and most widespread of the *Forastero* cocoa. When ripe, the pods are hard and each contain around 40 beans. The shape of the pods is different from the *Criollo*, being more rounded and melon shaped (giving the origion of the name *Amelonado*). The beans are smaller and ranging from pale to dark purple and they have a stronger flavour. The *Trinitario* (classified by botanists as a hybrid between *Forastero* and *Criollo* types) is less susceptible to diseases and intolerant to drought and produces cocoa with special flavours (Afoakwa, 2010; Rusconi and Conti, 2010).

1.4 From tree to factory

Cocoa has a long supply chain. Farmers produce fermented beans, warehouses store beans, processors turn this into cocoa products, traders ship mainly to North America and Europe and manufacturers convert this into consumable products. The “first world” dominates the commodities market that determines the price of cocoa for the “third” world farmers (Schwan and Wheals, 2004). Figure 1.1 represents the flow chart starting from the

ripe pod to the production of cocoa mass, butter and powder. The different steps of this flow chart will be explained in the following sections.

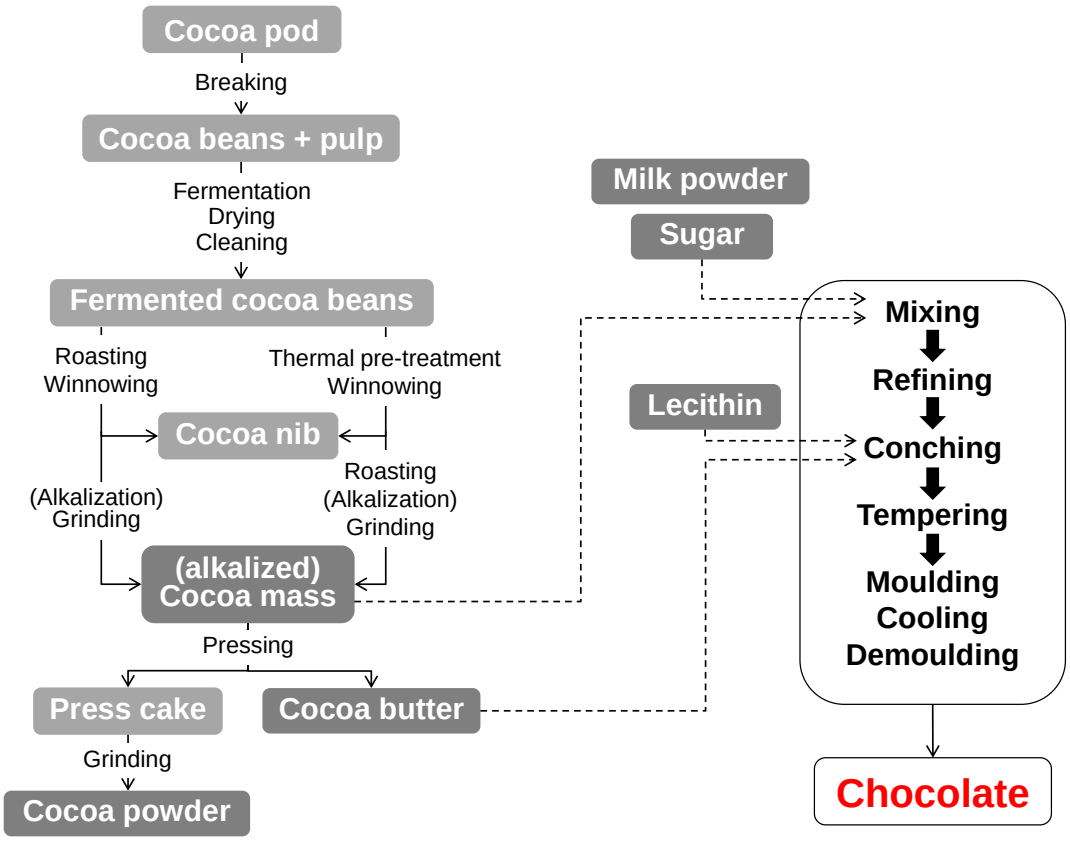


Figure 1.1 The cocoa processing and chocolate manufacturing chain

1.4.1 Harvest

After five years, the cocoa tree starts to develop thousands of tiny white (female) and pink (male) flowers of which no more than 40 develop into cocoa pods. The cocoa pods are ripe over a period of 6 months during which they change colour: from green/red to yellow/ orange. The pods are cut by hand with knives. There are two harvest periods per year: the main-crop is harvested from October to March and usually has a higher quality than mid-crop, harvested from May to August. Farmers open the outer peel by cutting or cracking with a wooden club to extract the fruit pulp inside. The pulp contains 30 – 50 of seeds, the precious cocoa beans, attached to a placenta. Pulp and seeds are separated by hand and the placenta is removed. The beans consists of two cotelydons, a germ enclosed in a skin (the shell). The cotelydons serve as storage organs, containing of 50% of cocoa butter on a dry base. The moisture content of the fresh beans is in the region of 65% (Barel, 1998; Dand, 1999; Fowler, 2009).

1.4.2 Fermentation

To start the fermentation and allowing natural yeasts and bacteria to multiply in the pulp, the beans and pulp are heaped in a pile covered with banana leaves or in a wooden box. The process usually lasts about 5 to 7 days and the end point is often determined by experience. The duration of the fermentation is depending on the country, cocoa variety and on the farmers' practice. Variation in the fermentation period will lead to different end products with specific characteristics. A fermentation period of four days, for example, will result in an acid cocoa as the concentration of acetic acid reaches its maximum.

Figure 1.2 gives a schematic presentation of the chemical changes occurring during the fermentation. One should make the distinction between two types of reactions taking place:

- fermentation reactions in the pulp
- biochemical reaction in the cotyledons of the beans.

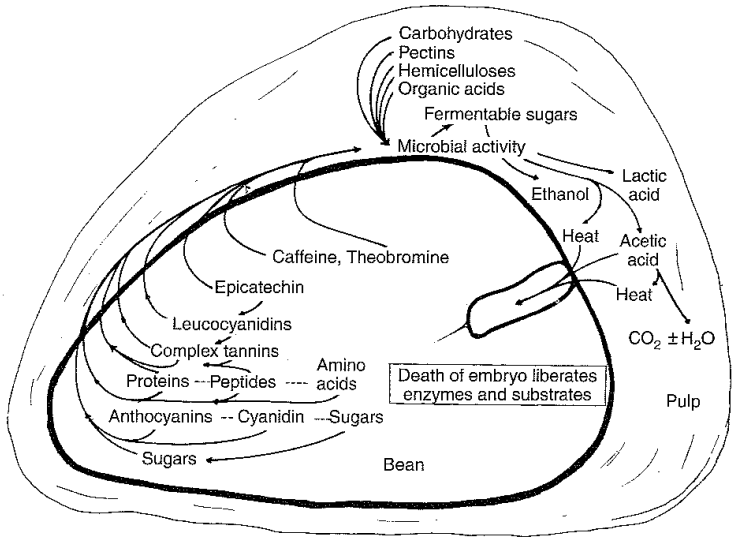


Figure 1.2 Chemical changes within a cocoa bean during fermentation (Fowler, 2009)

As the pulp contains mainly water (80%), sugar, especially glucose (12-15%), pectines and acetic acid, it forms an ideal growth medium for a variety of micro-organisms that are naturally introduced from the environment when the pods have been opened. The fermentation process can be divided into three stages. In the first 24 – 36 hours, conditions are favourable for anaerobic yeasts which convert sugar into alcohol in an exothermic process (temperature increase is limited). Bean death occurs on the second day, usually caused by acetic acid and alcohol. Some yeast secrete pectinases. The pulp turns liquid and is drained away. At the same time air is allowed to enter the heap. This announces the start of the aerobic phase. To improve the aeration in this phase, farmers often mix the beans by transferring them to another box. Lactic acid bacteria become dominant between 48 – 96

hours. They convert sugar and some organic acids into lactic acid. The formation of lactic acid is not really desired as it enters the cotyledons to remain there definitely, leading to an acid cocoa of inferior quality. In a good fermentation process, the acetic acid bacteria should be dominant. They transform alcohol into acetic acid in an strongly exothermic reaction, increasing the temperature to 50°C. The succession of the anaerobic – aerobic phase is essential for a good fermentation.

At the same, time key changes occur in the cotyledons of the beans. After bean death, cell walls and membranes of the storage cells (containing fat and protein) and the pigment cells (containing polyphenolic compounds and methylxanthines) break down, allowing various compounds and enzymes to react. In this way the precursors of the chocolate aroma are formed. Methylxanthines and flavonoids, imparting for bitterness and astringency respectively, decrease significantly. Anthocyanins are hydrolysed to cyanidins and sugars, which accounts for bleaching or lightening of the purple colour in Forastero cocoa. Flavanols (mainly epicatechine) are converted by polyphenoloxidase to quinones which on their turn interact with proteins and peptides to form brown/purple coloured components.
An important group of compounds are the Maillard reaction precursors. Storage proteins are transformed to oligopeptides and amino acids by peptidase enzyme action. Sucrose on the other hand is converted by invertase into reducing sugars. The Maillard reaction is important during the roasting process were cocoa flavour compounds are formed (Afoakwa, 2010; Barel, 1998; Fowler, 2009).
The microbial activity and the formation of the flavour precursors during fermentation have been extensively described by different authors (Afoakwa et al., 2008a; Camu et al., 2008a; Camu et al., 2008b; Guehi et al., 2010; Lefeber et al., 2010; Schwan and Wheals, 2004).

1.4.3 Drying and storage

To improve the conservation of the beans, the moisture content should be reduced from 55-60% to 7% by drying. Flavour development from cocoa beans precursors continues during drying together with the development of a characteristic brown colour. Major polyphenol oxidizing reactions are catalyzed by polyphenol oxidases, giving rise to new flavour components, and loss of membrane integrity, inducing brown colour formation (Afoakwa et al., 2008a). Where the weather is dry and sunny during the harvest period, beans are spread out on mats, trays or a terrace and they are sun dried for about 7 to 15 days. When weather conditions are not optimal, improved methods of solar drying or artificial drying are necessary. Artificial drying introduces two problems: the drying may be too quick resulting in acidic beans as volatile organic acids are trapped by the hard shell. Secondly, smoke may

find its way to the bean resulting in a harsh, smoke or tar taste which cannot be removed during the processing. It is also important not to dry too much as beans with a moisture content below 6% become brittle and are easily damaged during handling.
After drying it is necessary to carry out a manual sorting or mechanical sieving to remove debris and broken beans before they are bagged in jute sacks. The bags should be stored in warehouses with good ventilation to prevent moisture uptake. Cocoa beans are susceptible to tainting by uptake of certain odorous substances from other commodities, chemicals so the presence of the latter should be avoided (Barel, 1998; Fowler, 2009).

1.4.4 Cleaning

Before further processing, the beans are always cleaned to remove non-cocoa components like stones, pieces of metals, debris. Impurities may damage the machinery used in subsequent processing. A set of vibrating screens removes both very large and very small particles by sieving. Counter air flow techniques removes low density and dust particles. Ferrous material is removed by magnets (Kamphuis, 2009).

1.4.5 Cocoa processing and technology

1.4.5.1 Removal of the shell

The removal of the shell is mandatory as the shell may contain undesirable contaminants and could give off-flavours to the final product. The shell represents 10 – 12% of the total bean weight (De Ginestel, 1998). Ideally, the shell separates perfectly leaving large pieces of shell and almost the intact nib. In practice beans are usually subjected to surface heat treatments to facilitate the shell release afterwards. Fluid bed type of dryers, continuous air roasters, infra-red dryers or moistening/pre-drying systems can be used for this purpose (Kamphuis, 2009).

1.4.5.2 Breaking and winnowing

In a swing-hammer breaker the beans are broken due to centrifugal forces. The distribution should be as homogeneous as possible for optimum separation of shell and nibs. In the next step, the winnowing, broken beans are divided into a number of fractions by means of sieves and an air classifier separates the shell from the nib particles (Kamphuis, 2009). The shell itself is used for animal feed or may end up in the local garden centre.

1.4.5.3 Alkalization

In the alkalization process, the nibs are treated with an alkali solution (usually potassium carbonate) to obtain a mass with a higher pH, a darker colour and a typical taste. Alkalization is predominantly used to produce cocoa powders with specific colours (varying from brown, red and even black) used in drinks, bakery product and coating. But alkalized cocoa masses may also be used in chocolates to introduce specific flavours. The colour is depending on the type of beans and process conditions like time, temperature, pressure, amount of alkaline solution. Therefore the alkalization process requires a lot of experience and skill in order to obtain products with consistent colour in combination with good flavour characteristics (Camu et al., 2008a; De Ginestel, 1998; Kamphuis, 2009; Rodriguez et al., 2009).

1.4.5.4 Roasting

Roasting of cocoa is an essential step to further develop the chocolate flavour from the precursors formed during fermentation and drying. It brings about formation of the characteristic brown colour, mild aroma and texture of roasted beans (Krysiak, 2006). Maillard reactions, central to cocoa flavour development, are important in roasting as free amino acids, peptides, and reducing sugars all participate (Afoakwa et al., 2008a). Whole bean roasting loosens the shell which is then readily removed in winnowing. This was the original method which was often used to produce cocoa masses with delicate flavours due to the preservation of volatile flavours within the shell during roasting. However, the main disadvantage of this process is that fat migrates from the nib to the shell, thus reducing the fat content of the obtained cocoa mass. Additional disadvantages are that the shell is also heated, which may cause combustion gases from any remaining foreign materials on roasting of the shell to affect the flavour of the cocoa mass. Furthermore, the whole bean roasting process is never optimal for all beans because of their different sizes. Due to all these disadvantages, this traditional process of whole bean roasting gradually disappears in favour of nib roasting (De Ginestel, 1998).

Many different roasting systems are available in which conduction and/or convection are used as the heat transfer mechanism. Batch drum roasters are frequently used in the cocoa industry for nib roasting. Effective debacterization can be carried out by adding water and assuring the presence of steam in the drum (Kamphuis, 2009). The degree of cocoa roast shows a time/temperature dependent relationship, over periods of 5 to 120 min and in the range 120 to 150°C (Afoakwa et al., 2008a).

1.4.5.5 Grinding

The last step in the production of cocoa mass is grinding. The fineness to which the mass needs to be ground depends on its final use.

1.4.5.6 Cocoa, a natural medicin?

Cocoa and chocolate products have recently attracted the attention of many investigators and consumers because of their potential medicinal and mystical properties (Afoakwa, 2008). Over the past few years, the antioxidant and health-promoting properties of cocoa and cocoa-related products have been thoroughly investigated and reviewed (Arlorio et al., 2008; Cooper et al., 2008; Corti et al., 2009; Galleano et al., 2009; Rimbach et al., 2009; Rusconi and Conti, 2010). Polyphenols, widely distributed in plant foods, are the main antioxidant-active fraction of cocoa, and, within polyphenols, flavanols and procyanidins have been identified as the active antioxidant agents of cocoa and dark plain chocolate. While various monomeric [(−)-epicatechin, (+)-catechin, (+)-gallocatechin, (−)-epigallocatechin and epicatechin 3-*O*-gallate] and oligomeric (3 – 11 units) flavan-3-ols from cocoa show powerful antioxidant activity the contribution of other polyphenolics like flavonoids (quercetin, quercetin glycosides, luteolin, naringenin, apigenin) and anthocyanidins has not yet been fully assessed (Arlorio, 2008). At present, the wide variation in cocoa processing and in the content and profile of polyphenols make it difficult to determine to what extent the findings about positive effects expressed in different studies, translate into tangible clinical benefits (Rusconi and Conti, 2010). Therefore, the consumption of cocoa and chocolate products still presents an exciting area of further nutritional, clinical and epidemiological research (Afoakwa, 2008).

1.4.6 Cocoa butter extraction

Cocoa beans contain up to 54% of cocoa butter. This fat can be extracted from various raw materials leading to five different qualities of butter. Typically, 100 g of beans produce 40 g of cocoa butter, 40 g of cocoa powder (the residue after extraction which contains 10 – 24% fat) and 20 g of waste material (shell, moisture, dirt) (Timms and Stewart, 1999).

Although the process used to separate the cocoa butter is no longer regulated, the Federation of Cocoa Commerce Market rules divide cocoa butter into press cocoa butter, expeller cocoa butter and refined cocoa butter.

The best quality, pure press butter, is extracted with hydraulic presses, from the cocoa liquor made from good quality nibs. In industrial production, cocoa mass is pumped into the press chamber at 1.5 – 2 MPa. Once the press chamber is filled the pressure is linearly increased

to the required end pressure. The applied pressure is kept constant for approximately 10 min before the filter cake is removed. Industrial hydraulic presses consist of twelve or more filter pots with an inner diameter of 425 – 600 mm, and operates invariably at temperatures of 90-110°C to optimize the yield. The filter cake is then broken into small pieces, grinded and sold as cocoa powder. The price of cocoa butter is almost double than that of cocoa powder (Venter et al., 2007).

Expeller cocoa butter is obtained by physical removal from cocoa beans, nibs, mass, press cake or any combination of these, using a so-called expeller. Pressing cocoa nibs may overcome the entrainment of solid particles into cocoa butter, clogging of the filter medium in the press chambers and malfunctioning of the sealing ring used in the press chambers may occur during the pressing of cocoa mass. But Venter et al. (2007) concluded that the pressing of nibs leaded to lower yields (80%) compared to the pressing of cocoa mass (89%).

Refined cocoa butter includes press, expeller and solvent extracted cocoa butter that has been refined by neutralization with an alkaline solution and decoloured with for example betonite or activated carbon (Kamphuis, 2009).

The expeller cake and various cocoa waste materials are completely defatted by solvent extraction with hexane. Increasing awareness of health and safety hazards associated with the use of organic solvents, due to its possible contamination of extracted products, has placed new demands. Therefore supercritical fluid extraction using carbon dioxide and ethane were used as an alternative by Saldana et al. (2002) to extract cocoa butter from Brazilian cocoa beans.

1.5 Chemical properties

The chemical composition and characteristics of cocoa butter are greatly influenced by the variety of cocoa and the growth conditions. As a consequence there exists a lot of variation in the properties of cocoa butter, not only between the varieties but also within one variety.

1.5.1 Fatty acid composition

In terms of fatty acid composition, cocoa butter is a relatively simple fat. The main fatty acids (95% of total) are palmitic (P, C16:0), stearic (St, C18:0) and oleic (O, C18:1) acid. Next to these fatty acids, also linoleic (L, C18:2) and arachidic acid (A, C20:0) are present in considerable amounts. Palmitic acid, stearic and oleic acid account on average for respectively 26%, 36% and 34% of the total FA composition (Foubert et al., 2004b; Talbot, 2009b).

1.5.2 Triacylglycerol composition

This “simple” fatty acid composition results in a “simple” triacylglycerol (TAG) distribution which mainly consists of symmetrical mono-unsaturated triacylglycerols: 1,3-dipalmitoyl-2-oleoyl-glycerol (POP), *rac*-palmitoyl-stearoyl-2-oleoyl-glycerol (POSt) and 1,3-stearoyl-2-oleoyl-glycerol (StOSt) accounting on average for respectively 18%, 41% and 25% of total TAG composition (calculation based on Foubert et al. (2004b)). Each of these TAG is an individual chemical entity with a particular melting point and the combination in cocoa butter imparts the sharp melting profile for which cocoa butter is prized (Talbot, 2009b). Apart from these mono-unsaturated TAG, some di-unsaturated TAG and a very small amount of tri-unsaturated TAG are present. The TAG composition is the main distinguishing factor between the different origins. An extensive amount of cocoa butter samples have been characterized by Chaiseri and Dimick (1995), Foubert et al. (2004b), Lipp et al. (2001) and Shukla (1995). The main difference between cocoa butters from different countries of origin is the ratio of SOS/SOO (Table 1.1).

Table 1.1 Triacylglycerol composition of cocoa butters (Talbot, 2009c)

	Brazil	Ghana	Malaysia
SSS	1.0	1.4	2.3
SOS	63.7	76.8	84.0
SSO	0.5	0.4	0.5
SLS	8.9	6.9	6.8
SOO	17.9	8.4	5.1
OOO	8.0	6.1	1.3

S= saturated fatty acids (mainly palmitic and stearic acid); O= oleic acid; L= linoleic acid

Brazilian butter and also other South American butters contain higher amounts of di-unsaturated TAG while the Asian butter shows the lowest amount of these type of TAG. In very broad terms, SOS is the group of TAG that is solid at room temperature whereas the SOO type of TAGs are more liquid at room temperature. So, Brazilian butter can be classified as a soft butter and the Malaysian as a hard butter. The African butters usually show intermediate behaviour (Talbot, 2009b; Timms, 2003). These compositional effects also influence the crystallization kinetics as Chaiseri and Dimick (1995) concluded that soft cocoa butter is usually associated with a slow crystallization rate and hard butters show rapid crystallization.

1.5.3 Minor Components

Besides TAG, cocoa butter contains some other components in much smaller amounts. These so-called minor components include free fatty acids, mono- and diacylglycerols (DAG), phospho- and glycolipids and unsaponifiable matter. These minor components are of importance as they may influence crystallization (Chaiseri and Dimick, 1995).

Foubert et al. (2004b) investigated the composition of 20 different cocoa butter. The amount of free fatty acids diverged from 1.16% for a hard cocoa butter to 2.77% for a Nigerian cocoa butter. The amount of diacylglycerols (expressed on TAG) varied between 0.6 to 2.2% again depending on the origin of the cocoa beans. These results are in accordance with other studies (Chaiseri and Dimick, 1989; Shukla, 1995). For phospholipids, Chaiseri and Dimick (1995) and Foubert et al. (2004b) reported lower values (0.1 to 0.2%) than Shukla (1995) and Savage & Dimick (1995) who found values of 0.7 to 1.0%

The amount of unsaponifiable matter, including higher aliphatic alcohols, sterols, pigments and hydrocarbons, depends on the author cited: values range from 0.3 to 1.5% (Foubert, 2003). The oxidative stability of the cocoa butter is an important characteristic which is also affected by its chemical composition. Copper and iron are prooxidants meaning that they induce the oxidation of the cocoa butter. α-tocopherol, on the other hand, is known as an antioxidant, although at higher levels it can behave as a prooxidant (Hashim et al., 1997).

1.5.4 Quality parameters

High quality raw material is needed to make the best quality cocoa butter. Press butter, which is commonly used in chocolate industry, is only filtered and (partly) deodorized to obtain the desired flavour. Table 1.2 lists the quality parameters for pure press cocoa butter.

Table 1.2 Quality parameters for cocoa butter

Parameter	Value
Free fatty acids	Max 1.75%
Iodine Value	33 – 42
Refractive index	1.456 – 1.459
Moisture Content	Max 0.1%
Unsaponifiable matter	Max 0.35%
Blue value	Max 0.05%

For expeller and refined cocoa butter, the threshold value for free fatty acids is the same but it is increased to 0.50% for unsaponifiable matter. This higher threshold for unsaponifiable

matter in expeller and refined cocoa butter is due to the fact that fat obtained from the shell contains more unsaponifiable matter (European Union, 2000; Kamphuis, 2009).

1.6 Physical properties

Due to its typical chemical composition cocoa butter has unique physical properties. First of all it is strongly polymorphous fat. But the most remarkable is the very narrow melting range, between 32-35°C, just below body temperature. In chocolate this is translated in a very quick meltdown in the mouth, resulting in a cool sensation and good flavour release.

1.6.1 Polymorphism

Polymorphism, derived from the Greek words *poly* (many) and *morphos* (form), describes the multiple-melting point behaviour. This is due to the fact that a TAG molecule can crystallize in a number of different crystal packing configurations (Timms, 2003). The "three legged" molecules can fit together in different ways, like stacking chairs. There are two ways, a double-chain packing and a triple-chain packing (Figure 1.3a). These small stacks fit together with other stacks. The angle at which they fit together determines their stability. The differences between polymorphs are the most apparent from a top view of the planes which show the subcell structure. Based on this, three polymorphic forms can be defined: α, β' and β, in this order of increasing stability and melting point (Figure 1.3b). These structures can be defined by X-ray powder diffraction (Himawan et al., 2006; Sato, 2001).

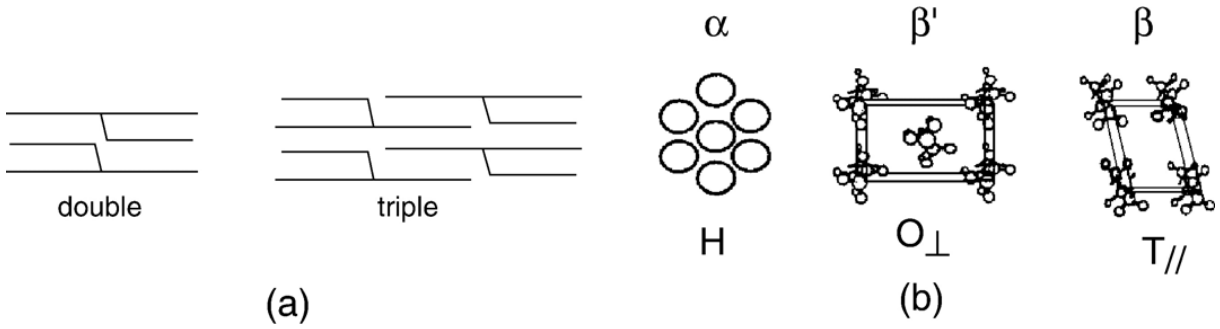


Figure 1.3 The three most common polymorphs in TAGs (a) chain-length packing structures and (b) subcell structures (Himawan et al., 2006)

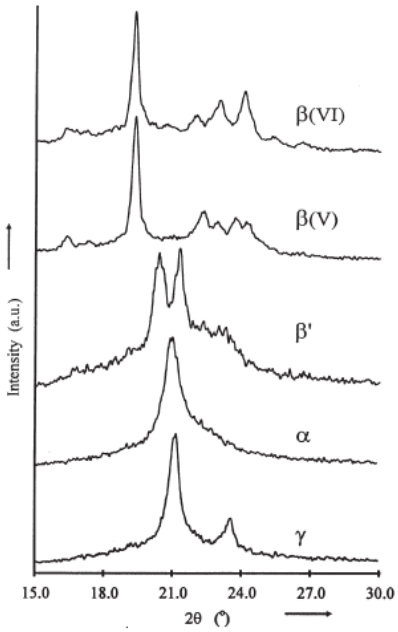
While polymorphism is present in most fats, those rich in SUS type of TAG with oleic acid at the sn-2 position (like cocoa butter) are highly polymorphic. The presence of a double bond (with the inflexible "kink") give more steric hindrance, forcing specific structures to be formed

which enable that the saturated and unsaturated fatty acid moieties are packed together in the same lamella leaflet (Himawan et al., 2006).

The polymorphic behaviour of cocoa butter has been the subject of a lot of research but up to the present day the number and type of polymorphs has still been subject of controversy. Traditionally, the confectionery industry has considered the polymorphism of cocoa butter to be that as defined by Wille and Lutton (1966) in which the different forms are named the Roman numerals from I to VI. However, an earlier study of Vaeck (1960) on fat bloom only distinguished five polymorphs. In 1971, Huyghebaert and Hendrickx were able to define the six polymorphs by using differential scanning calorimetry (DSC). However, Merken and Vaeck (1980) found no more than four polymorphs not only claiming the absence of forms III/IV but also the existence of form VI. The latter was defined as formed by phase separation. The discussion continues as van Malssen (1996) again reported that forms III and IV did not exist independently. All polymorphs could be formed from the liquid, except the β_V and β_{VI} which are obtained by transformation from β'. Forms β_V and β_{VI} were considered as different sub phases. But in 1999, van Malssen et al. confirmed the presence of two distinct forms β_V and β_{VI}. The β' was still considered as a phase range rather than as two separated phases, III and β_{IV}. Timms (2003) concluded from all the studies that β_V and β_{VI} are genuinely different polymorphs and that X-ray diffraction (XRD) short spacings can unequivocally distinguish six different polymorphs. An overview of the melting temperature or range as reported by different authors is given in Table 1.3. Figure 1.4 shows the typical WAXD reflections and short spacings values of the different polymorphs.

Table 1.3 Melting temperature (range) of cocoa butter polymorphs as reported by different authors

Phase		Melting point (°C)			
Wille (1966)	Windhab (2009)	Wille (1966)	Huyghebaert (1971)	Lovegren (1976)	Van Malssen (1999)
I	γ	17.3	14.9 – 16.1	13	-5 – 5
II	α	23.3	17 – 23.2	20	17 – 22
III	III	25.5	22.8 – 27.1	23	20 – 27
IV	β_{IV}	27.5	25.1 – 27.4	25	
V	β_V	33.8	31.3 – 33.2	30	29 – 34
VI	β_{VI}	36.3	33.8 – 36.0	33.5	



Polymorphic form	Short spacing (Å)*
γ (sub-α)	3.87 (m), 4.17 (s)
α	4.20 (vs)
β_2'	3.87 (vW), 4.20 (vs)
β_1'	3.75 (m),3.88 (w), 4.13 (s), 4.32 (s)
β_2	3.65 (s), 3.73 (s), 3.87 (w), 3.98 (s) 4.22 (w), 4.58 (vs), 5.13 (w), 5.38 (m)
β_1	3.67 (s), 3.84 (m), 4.01 (w), 4.21 (vw), 4.53 (vs), 5.09 (vw), 5.37 (m)

*The relative intensity is noted as very strong (vs), strongs (s) medium (m), weak (w) and very weak (vw)

Figure 1.4 Characteristic Powder XRD Wide Angle reflections (short spacing) of the various polymorphic forms of cocoa butter (Marangoni and McGauley, 2003 and Van Malssen et al., 1999)

Figure 1.5 gives a schematic overview of the phase transitions in cocoa butter. γ, α and β' phases can crystallize directly from the melt. The α to β' transformation is much quicker than directly from the melt. The two β phases, V and VI are only obtained via a phase transformation from the β' phase (van Malssen et al., 1999).

Well-tempered chocolate is crystallized in form β_V, which results in the appropriate chocolate properties. Due to a long storage time and temperature fluctuations, this β_V form can transform into β_{VI} which can be visually detected as fat bloom (Talbot, 2009a).

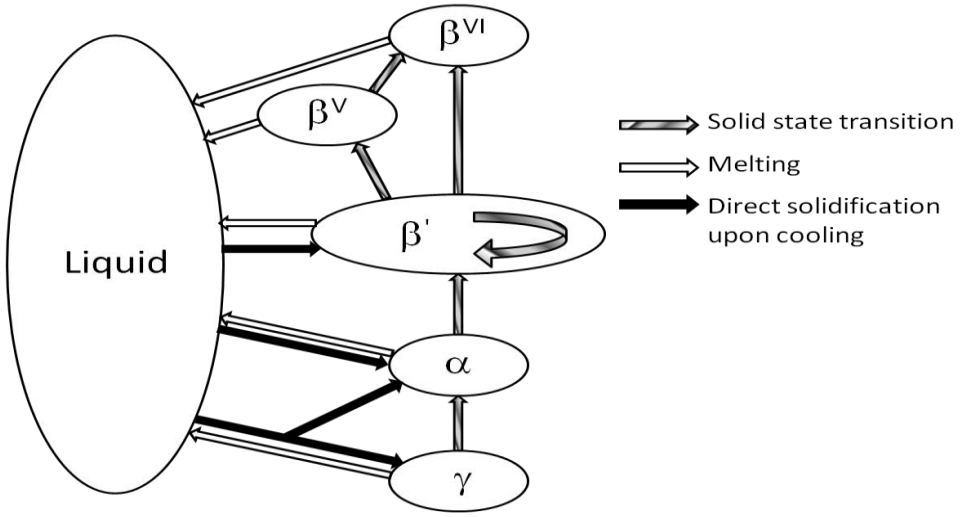


Figure 1.5 Cocoa butter phase transition scheme as proposed by Van Malssen et al. (1999)

1.6.2 Crystallization

Crystallization may be defined as a first order transition of an ensemble of molecules from the liquid state to the solid state in such a manner that the molecules within the solid state pack in regular repeating manner to form a solid lattice (Vereecken, 2010). It plays a critical role in determining the sensorial properties and stability of many food products including chocolates and confectionary coating, dairy products, vegetable spreads, etc. (Dewettinck et al., 2004). Insight in the different processes involved in fat crystallization is important to evaluate the final product structure, functionality and quality. Three levels can be identified: a nano-, micro- and macro-scale, as shown in Figure 1.6. The primary crystallization (nanoscale) is characterized by nucleation and crystal growth and is followed by aggregation into clusters and network formation. These different steps are not discussed in detail as a broad range of good literature reviews on this topic are available (Garside, 1987; Himawan et al., 2006; Sato, 2001).

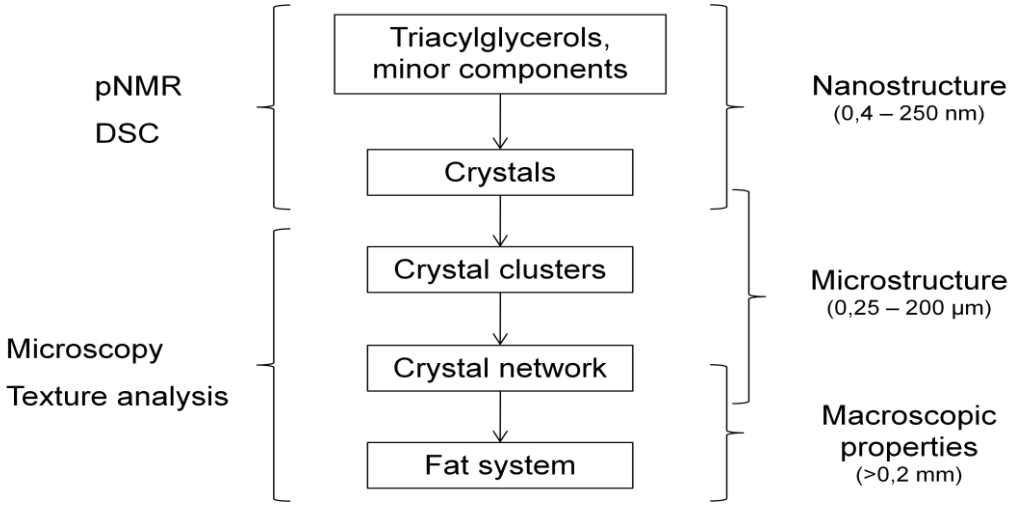


Figure 1.6 Structural hierarchy in fatty materials (Narine and Marangoni, 2005)

The crystallization of fat is often described under isothermal conditions. Marangoni & McGauley (2003) and Van Malssen et al. (1999) constructed an time-temperature state diagram for the polymorphism of statically crystallized cocoa butter based on mainly DSC and XRD data respectively. The diagram of Marangoni and McGauley (2003) is given in Figure 1.7. They concluded that below -15°C a mixture of the transient metastable γ and α phases was formed. Between 15 and 20°C, the material nucleated initially into an α form and then gradually transformed to the β'. This transition is completed faster at higher temperatures. At temperatures from 21 – 26°C, formation of the β' takes place directly. The β polymorph was only formed via the β' from.

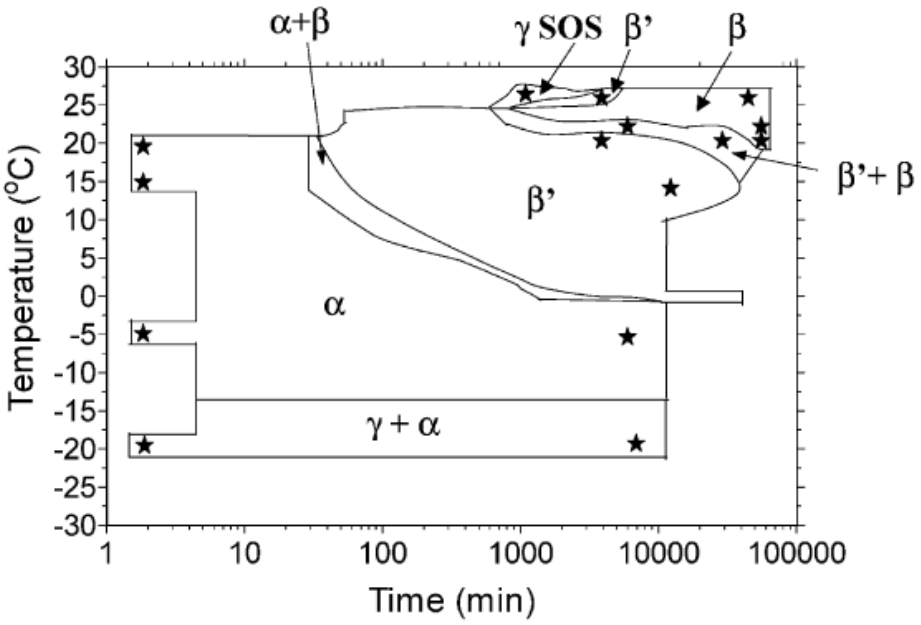


Figure 1.7 Time-temperature state diagram for the polymorphism of statically crystallized cocoa butter (Marangoni & McGauley, 2003)

The crystallization kinetics of fats (particularly the rate of crystallization and the rate of change from one polymorph to another) are important as the equilibrium behaviour of fats and their mixtures since they are relevant to real systems of fat production (Foubert et al., 2003).

Several experimental techniques can be used to follow the isothermal crystallization of fats as function of time. Differential scanning calorimetry (DSC), pulsed nuclear magnetic resonance (pNMR), transmittance/turbidity measurements, time-resolved XRD, polarized light microscopy (PLM), rheometry and ultrasound velocity measurement (Foubert et al., 2003).

Many researchers have tried to model the isothermal crystallization behaviour of cocoa butter. The Avrami model is most commonly used to describe the isothermal crystallization kinetics of fats. Some authors have also used a modified Avrami equation or an reparameterized Gompertz equation to describe the crystallization curves (Foubert et al., 2003). Foubert et al. (2002) developed an alternative model, available in both algebraic and differential equation form based on cocoa butter crystallization kinetics. According to Foubert, the heat release or the solid fat content (SFC) during a single-step isothermal crystallization is described by the following algebraic function as function of time t:

$$f(t) = a_F\left(1 - \left(1 + \left(0{,}99^{(1-n)} - 1\right)e^{\left((n-1)K(t-t\_ind)\right)}\right)^{\left(\frac{1}{1-n}\right)}\right)$$

This model can be fitted to the integral of the main crystallization peak observed in DSC or to the sigmoid increase of SFC as measured by pNMR yielding the four Foubert parameters: namely t_{ind}, K, a_F and n. The induction time, t_{ind} (h) is defined as the time needed to obtain x% of crystallization, x is chosen to be 1, K (h^{-1}) is the rate constant, a_F (Jg^{-1}) is the maximum amount of crystallization and n [-] is the order of the reverse reaction.

Temperature is an important factor in the crystallization of cocoa butter. A higher temperature leads to a higher t_{ind}. The rate constant, K, decreases as temperatures increases. The order of the reverse reaction (n) decreases until a temperature of 20.5°C to 21.5°C, once this temperature is reached the order remains constant. The equilibrium amount of solid fat decreases with increasing temperature (Foubert et al., 2003).

A change in chemical composition may cause a difference in crystallization kinetics. The SFC and the induction time are mainly influenced by the TAG composition. Chaiseri and Dimick (1989) showed that the solid fat content depends on the amount of di-unsaturated TAG present in the fat: the higher the di-unsaturated TAG amount, the softer the fat. Fats with a higher concentration of POO and StOO and a lower concentration of POSt and StOSt have a longer induction time (Chaiseri and Dimick, 1995).
In further research, Foubert et al. (2004) investigated the influence of the chemical composition of cocoa butter on the crystallization characteristics. They concluded that the ratio of saturated to unsaturated fatty acids and mono-unsaturated to di-unsaturated TAG influence a_f, t_{ind} and n to a great extent. Furthermore, a_f is influenced negatively by the FFA content and the DAG content. t_{ind} increases with an increasing amount of DAG, phosphorus and FFA. Besides this, n is positively influenced by the peroxide value and the phosphorus content, while it is negatively influenced by the iron content and DAG. The rate constant K is particularly influenced by the amount of DAG, FFA and soaps.

1.6.3 Melting behaviour

The melting of cocoa butter takes place from 15 to 36°C, depending on the polymorphic form (Huyghebaert and Hendrickx, 1971). The solid fat content (SFC) is the amount of solid fat present in a fat. The SFC curve of CB is characterized by typical zones, as shown in Figure 1.8. The SFC below room temperature (25°C) is an indication of the hardness of the fat. The heat resistance of a fat can be deduced from the solid fat present between 25°C and 30°C. If the fat has a relatively high solid fat content at temperatures above 37°C (body temperature), it can cause a waxy mouth feel (Talbot, 2009b; Torbica et al., 2006). The steepness of the melting profile of coca butter contributes to the flavour release. Because of

its characteristic melting profile, the flavour is released in a relative short time leading to an intense flavour. Next to the flavour release, the sharp melting profile is responsible for the cooling sensation in the mouth, due to the heat absorbed by the melting fat (Smith, 2001).

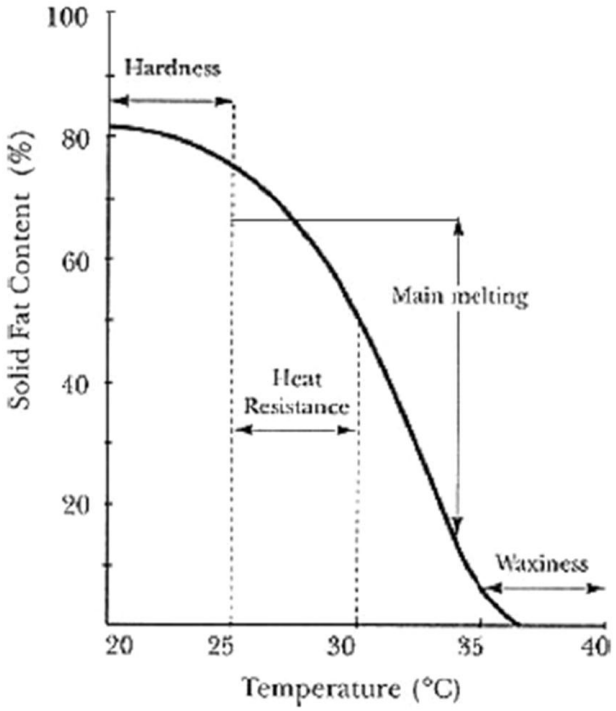


Figure 1.8 Cocoa butter SFC curve depending on the temperature (Torbica et al., 2006)

1.7 Chocolate production

Chocolate is semi-solid suspension of approximately 70% fine solid particles from sugar and cocoa (and milk powder depending of the type), in a continuous fat phase (Afoakwa, 2010).

1.7.1 Legislation

Directive 2000/36/EG of the European Parliament and the Council lays down characteristics for dark chocolate, milk chocolate and white chocolate.

Dark chocolate

- Minimally 18% cocoa butter
- Not less than 35% total dry cocoa, minimally 14% dry non-fat cocoa solids

Milk chocolate:

- not less than 25 % total dry cocoa solids, minimally 2.5 % dry non-fat cocoa solids
- not less than 14 % dry milk solids obtained by partly or wholly dehydrating whole milk, semi- or full-skimmed milk, cream, or from partly or wholly dehydrated cream, butter or milk fat, minimally 3.5 % milk fat
- not less than 25 % total fat (cocoa butter and milk fat).

White chocolate

- not less than 20% cocoa butter
- not less than 14% dry milk solids obtained by partly or wholly dehydrating whole milk, semi- or full-skimmed milk, cream, or from partly or -wholly dehydrated cream, butter or milk fat, of which not less than 3.5 % is milk fat.

1.7.2 Ingredients and recipes

Typical recipes for the production of milk, dark and white chocolate are given in Table 1.4. Sugar, cocoa mass, cocoa butter and lecithin are the four main ingredients of plain chocolate. Instead of cocoa mass, also cocoa powder can be used. Other ingredients, for example milk powder, milk fat or vanilla can be added to the chocolate. A general production scheme of chocolate is shown in Figure 1.1.

Table 1.4 Recipes for dark, milk and white chocolate (personal communication AAK)

Ingredient	Dark chocolate (%)	Milk chocolate (%)	White chocolate (%)
Sugar	50	40	44
Cocoa mass	40	16	/
Cocoa butter	10	19	28
Full cream milk powder	/	20	20
Skimmed milk powder	/	5	8
Lecithin	0.4	0.4	0.4
Flavour	0.02	0.02	0.02
Total fat content	*32*	*33*	*33*

1.7.3 Mixing

In the first step, sugar, cocoa mass, cocoa butter (and depending on the product category milk fat and/or milk powder) are mixed together in a continuous or batch mixer to obtain constant formulation consistency (Afoakwa, 2010).

1.7.4 Refining

The particle size of the dispersed solid phase in chocolate, particularly that of the largest particles, must be sufficiently small so that the chocolate does not feel gritty and coarse when eaten. Traditionally, the European chocolate has a lower fineness (15 – 22 μm) compared to the North American one (20 – 30 μm) but due to globalization the differences start to blur (Ziegler and Hogg, 2009). In industry, the paste from the mixer is first fed into a two-roll refiner. The refiner consists of two cylinders, which turn in opposite directions to break the particles and meanwhile the fat will coat the surface of newly formed surfaces. The maximum particle size will vary between 100 and 150 μm.

The fine grinding is usually carried out on a five-roll refiner. A refiner consists of a number of hollow cylinders, which are temperature-controlled. A thin film of chocolate is attracted to the increasingly faster rotating rolls until it is scraped off the final roll by a stationary knife. The particles are reduced 5 – 10 times, resulting in a final product with a maximum particle size in the range 15 – 35 μm. Next to the particle size reduction, refining also certifies that the particles are distributed into the continuous phase (Afoakwa et al., 2007; Ziegler and Hogg, 2009).

1.7.5 Conching

Conching, derived from the Latin word for shell, resembled in the shape of the equipment, is regarded as the endpoint in the chocolate manufacture. The conching process can be divided into three phases: a dry phase, a pasty phase and a liquid phase.

The dry and pasty phase are especially important in the flavour development by reducing the water content and undesirable volatiles and by developing the desired flavours. The liquid phase, which starts by adding the remaining cocoa butter and emulsifier, contributes greatly to the rheological properties by coating the particles with fat and by homogenizing the chocolate.

During the dry conching phase, the feed material enters as a powdery material. Frequently a small amount of fat (approximately 1%) is added to the conche. Together with the mixing action and the rising temperature, the feed material is turned into a crumbly mass. At this stage, moisture can easily escape provided that the conche is well ventilated. Initial moisture content is around 1.6% and should end around 1%. Moisture has a thickening effect upon chocolate as for every 0.3% moisture left in the mixture (above a level of 1%), 1% extra fat must be added to compensate and retain the same viscosity. The moisture removal also has a major effect upon the taste of the chocolate by ‘steam distilling’ some of the flavour components. Indeed, cocoa mass, even when beans were correctly fermented, dried and roasted, has a very acidic flavour. Besides the volatile acids, mainly acetic acid, also some volatile aldehydes should be removed. During conching also a redistribution of the flavour components within the chocolate masse occurs. At the start, chocolate flavours exist only in the cocoa components (cocoa mass and cocoa butter) and sugar contains the sweet flavours. During conching, cocoa flavour components together with the fat are partially transferred to the sugar surface. This coating of the sugar particles results in a more uniform aroma perception. As this process continues, the crumbly chocolate is becoming more pasty. This starts with the formation of small balls on top of the masse. The viscosity will start to fall, part due to the moisture removal but also because many of the solid particles are now being coated with fat. This coating of the particle surface makes that they can flow past one another. In addition agglomerates, that may contain droplets of fats, should be broken by the mixing action. This fat is than released and smeared thinly over the particle surface.

In the last step, the actual viscosity should be reduced by adding more fat. The liquid phase can be very short but time should be sufficient for viscosity to reach an equilibrium. As cocoa butter has a high price, the goal is usually to find an optimal combination of cocoa butter-emulsifier to obtain the desired rheological behaviour. The emulsifier, lecithin, is cheaper but ten times more effective in reducing the viscosity compared to cocoa butter. One should also

take into account that lecithin is less efficient when added to chocolate with temperatures higher than 60°C (Afoakwa, 2010; Beckett, 2009b).

The chocolate flavour depends on the time and temperature used. Conching temperature varies with the type of chocolate: milk chocolate with whole milk powder has a conching temperature up to 60°C whereas the conching temperature of dark chocolate may vary between 70 – 82°C (Afoakwa, 2010). When conching milk chocolate above about 70°C, cooked flavour changes start occurring. Some manufacturers use temperatures above 100°C to try to promote some Maillard type flavours (Beckett, 2008).

Prawira and Barringer (2009) investigated the influence of an increasing conching time on particle size and sensorial properties of chocolate. They concluded that by increasing the conching time up to 76h, the particle size decreases and the chocolate has a more smooth mouthfeel.

Bolenz et al. (2003) put forward that with milk chocolate, flavour development is less important than developing the desired rheological and mouthfeel properties. Therefore, they suggest the use of shorter conching times when starting from high quality raw materials that contain as little water as possible.

1.7.6 Tempering

Since cocoa butter shows polymorphic behaviour, a conditioned crystallization process is required to obtain the desired polymorphic phase and its associated melting range (van Langevelde et al., 2001).

Tempering or pre-crystallization has two objectives (Timms, 2003):

1. To form nuclei and seed crystals (1 – 3%) total of the required polymorph so that during the main crystallization the chocolate will crystallize in this polymorph.
2. To form a large number of nuclei so that many small crystals form during the main crystallization.

Form β_V is the most desirable form giving well tempered chocolate the desired gloss, snap, contraction and resistance to bloom (Afoakwa, 2010).

The formation of a good crystal network is the result of a specific time-temperature process together with a shearing movement as illustrated in Figure 1.9. In a first step chocolate should be melted to 45 – 50°C to remove nuclei or other crystals. In the second step, supercooling takes place to induce crystallization (27 – 29°C) followed by a heating step above the melting point of the unstable α and β' crystals but below the melting point of the desired β_V (29 – 31°C). These melting points and temperatures vary according to the chemical composition of the cocoa butter i.e. its origin, the amount and composition of milk fat and the amount of any other StOSt-type fat present (Timms, 2003).

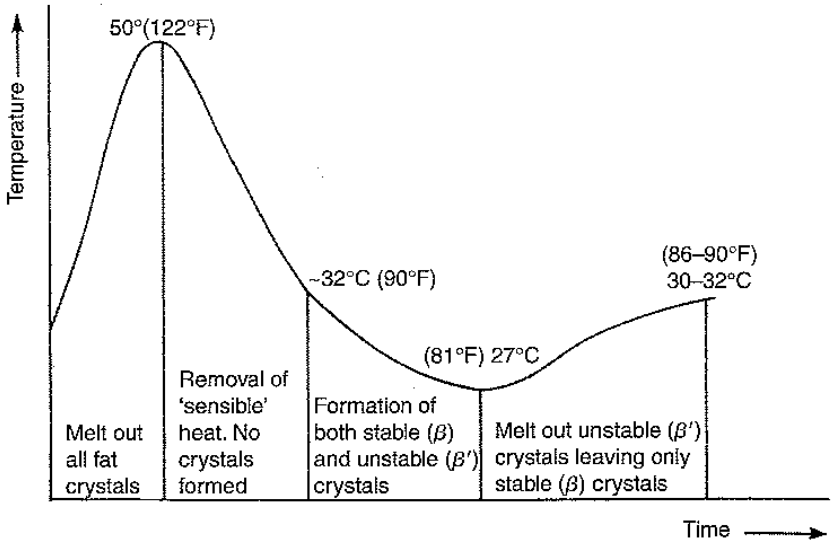


Figure 1.9 Temperature – Crystallization profile during pre-crystallization of chocolate (Talbot, 2009b)

Tempering can be done by hand on a marble plate for smaller quantities requiring a skilled person. In industry, automatic tempering units are used. Tempering machines are multistage heat exchangers. Time-temperature combination is important to create the right polymorph (Talbot, 2009a).

The state of temper refers to the amount of the desired polymorphic crystals. The amount and type of crystals can be measured directly by XRD, DSC or pNMR or more indirectly by a cooling curve. The latter is very useful for industrial practices and therefore a temper meter is often used. The temper meter consist of a temperature probe that punctures a disposable metal sample cup containing the tempered chocolate. The sample is then cooled by an ice-water bath or a thermo-electric cooler depending on the type of equipment. At the end of the measurement, a temper curve is obtained giving information on the degree of tempering. Typical temper curves are given in Figure 1.10. The first part is similar in all cases and simply registers the cooling of the liquid chocolate prior to solidification. The second part is the most defining state and is different depending on the state of tempering. When no or only a few crystals are formed, the chocolate cools to quite a low temperature before crystallization starts and a large temperature rise is seen because the bulk of the fat still has to crystallize releasing its latent heat. The chocolate is said to be under-tempered. In contrast, in over-tempered chocolate a large number of seed crystals are formed. The temperature at which crystallization starts is higher, less latent heat is released which can be more easily removed and so the temperature of the chocolate continues to decrease. In a perfectly tempered chocolate the rate of latent heat release is in balance with the heat removal and a plateau is observed. The degree of temper in CTU (Chocolate Temper Units) can be established from the position of the inflection point. In addition to CTU, the slope of

the curve and the temper index (TI) are two other parameters. The temper index is established from the position of the inflection point in the tempering curve and the slope. This value gives an idea about how the chocolate has been tempered (although it also depends on the type of chocolate). A high CTU value can be taken as an indicator for more stable crystals present (Afoakwa, 2010; Löser, 2009; Talbot, 2009a; Timms, 2003).

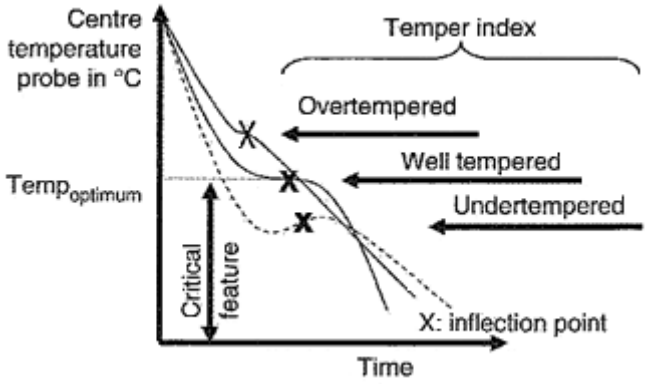


Figure 1.10 Typical temper curves from temper meter (Löser, 2009)

If chocolate is overtempered an increase in hardness and stickiness and a reduction in gloss and darkening of the surface is established. Fat bloom occurs if chocolate is under-tempered which leads to quality defects in texture, colour and surface gloss. Thus, optimal tempering is required to avoid quality defects affecting mechanical properties and appearance of products (Afoakwa, 2010).

1.7.7 Moulding, enrobing and cooling of chocolate products

The tempered chocolate can now be formed into a finished product by different ways. In moulding, the chocolate is poured in a mould for making solid tablets or for the formation of a chocolate shell in for composed chocolate products. In enrobing, a pre-formed centre is used over which chocolate is poured to produce a coating. The thickness and form of the coat are controlled by blowing off the excess mass using air circulation and then vibration of the product.

In both ways, subsequent cooling is required to solidify into the correct crystalline form. A large amount of latent heat and a relatively small amount of sensible heat needs to be removed by conductive or convective type of cooling (Gray, 2009).

1.8 Quality parameters of chocolate

Flavour, appearance and texture are three very important quality characteristics of chocolate. Those characteristics are defined by measurable variables like particle size distribution, rheology, hardness, crystallization behaviour, gloss and colour, which can be determined by means of instrumental techniques. The different steps of the production process will change these variables and subsequently result in the appropriate chocolate characteristics (Figure 1.9). Besides these instrumental techniques, sensory analysis can be performed to define human perception.

Table 1.5 Quality parameters influenced by different manufacturing steps of chocolate (Beckett, 2009c)

Manufacturing step	Quality parameters that are influenced
Production of the ingredients	Flavour
Recipe/mixing	Taste and flavour
Refining	Particle size distribution
Conching	Yield stress, viscosity, particle size distribution, flavour
Tempering	Melting properties, hardness, colour, gloss

1.8.1 Particle size distribution

The particle size distribution (PSD) is measured by a laser diffraction system where a laser beam will be scattered in angles that are related with the size of this particle. PSD has an influence on the rheological properties of chocolate (particularly the smaller particles) as well as on the sensorial properties (particularly the largest particles).

1.8.2 Flow behaviour

Chocolate viscosity and yield stress are very important parameters to control in chocolate manufacture, especially when chocolate is used for confectionary or ice cream coating. Adjusting the flow properties of molten milk chocolate allows the chocolate manufacturers to optimize their processes and formulations and to reduce the cost.

Cocoa butter exhibits Newtonian flow which means that the apparent viscosity is constant and thus independent of the applied shear rate. Chocolate is an example of a solid suspension, namely a polydisperse suspension of sugar, cocoa and/or milk solids in a Newtonian fluid (fat phase). Therefore, molten chocolate behaves as a non-Newtonian liquid, exhibiting a non-ideal plastic behaviour. This means that viscosity varies depending on the shear rate applied. At low flow rates the apparent viscosity is relatively high, while higher

shear rates provoke a lower viscosity. Beyond a certain value defined as the yield stress value, chocolate exhibits shear-thinning thixotropic behaviour. This means when that molten chocolate exhibits elastic behaviour and cannot flow if the applied stress level is lower than a yield value, whereas it flows when the stress reaches and overcomes this critical point. A transition from elastic to viscous deformation is then observed, which is due to the structure of the molten chocolate. Before the yield stress, solid particles interact together by chemical and mechanical interactions to form a network. Beyond the yield stress, the interactions disrupt progressively, which enhances the flow and decreases the viscosity (Ghorbel et al., 2011).

Flow behaviour can be expressed by fitting a mathematical equation to the flow curve (measured shear stress as function of the applied shear rate) for liquid chocolate. There are a large number of equations available but the Casson model is the most widely used within the chocolate industry. The Casson model has following basic equation:

$$\sqrt{\tau} = a\sqrt{\gamma} + b$$

$$\eta_{ca} = a^2$$

$$\tau_{ca} = b^2$$

in which: τ = shear stress (Pa), γ = shear rate (s^{-1}), η_{ca} = Casson viscosity (Pa.s), τ_{ca} = Casson yield stress (Pa).

The chocolate flow behaviour is important during processing as well as for the organoleptic aspects experienced by consumers. For the manufacturer correct flow behaviour is essential for processing conditions, such as pumping through pipelines, transportation of bulk chocolate. For the chocolate processors it is important to optimize their formulations depending on the application to ensure good moulding, enrobing and coating. The taste perceived by consumers depends upon the order and rate of contact, which is related by the viscosity and rate of melting (Afoakwa et al., 2007; Beckett, 2009a).
The rheological behaviour is mainly influenced by several factors such as fat content, PSD, surface active agents, the conching process and moisture content. Chocolate rheology was recently reviewed by Gonçalves et al. (2010).

1.8.2.1 Fat content

Adding more cocoa butter during the conching phase is a way to decrease the viscosity as the extra fat becomes part of the free fat phase and results in a better movement of the

particles. The effect of an extra 1% of fat upon the viscosity depends upon the amount already present. Above 32% fat, there is very little change in viscosity with any further addition. A 1% increase at 28% has a really dramatic effect on plastic viscosity. The effect on the yield stress is smaller as this parameter is determined by particle-particle interactions and thus the specific surface area. As fat is the most expensive ingredient, the main purpose is often getting a viscosity as low as possible with a minimum of cocoa butter (Afoakwa et al., 2007).

Milk chocolate is produced by adding milk to the recipe. This is done through whole milk powder or through low fat milk powder and anhydrous milk fat (AMF). The last option results in a higher free fat content. It was concluded that an increase in the ratio of milk fat on total fat will decrease the viscosity. This can be explained by the presence of low- and middle melting TAGs in AMF which cause an incomplete mixing of AMF and cocoa butter (Haylock and Dodds, 2009).

1.8.2.2 Particle size distribution (PSD)

PSD and rheological parameters are highly correlated. The yield stress increases when particle size decreases because more particles can interact. The effect on viscosity is much lower (Beckett, 2009a). It was observed that a bimodal PSD, i.e. a PSD with two peaks, will decrease the viscosity because the small particles fill in the spaces between the larger ones. This results in more particles per unit of volume and thus a higher yield stress (Ziegler and Hogg, 2009).

1.8.2.3 Surface active agents (emulsifiers)

Emulsifiers play an important role as they influence the viscosity by helping the hydrophilic sugar particles to be coated with fat. The most common emulsifier is lecithin. One end of the molecule is lipophilic and remains in the fat, whereas the other lipophobic end attaches itself to the sugar. When adding more than 0.5% the yield stress will increase due to micelle formation or the creation of double layers, reducing the effectiveness. Other emulsifiers which can be included are polyglycerol polyricinoleate (PGPR), citrem, soritan tristearate, sucrose ester and calcium-stearoyl lactoyl lactate (Beckett, 2009a).

1.8.2.4 Moisture content

Small amounts of water can raise the thickness of the chocolate significantly. A higher moisture content results in a higher viscosity. This is probably due to water on the surface of

the sugar particles which sticks them together and impedes the flow, but when lecithin is present this is less likely to happen as lecithin can have more water present (Afoakwa et al., 2007; Beckett, 2009a).

1.8.2.5 Conching

The conching process also has a large effect upon the final chocolate viscosity. Next to the time of addition of fat and emulsifier, also the time and intensity of conching determines the final viscosity of the chocolate. Intense and longer conching of the product gives rise to a thinner chocolate. Conching has the aim to remove moisture from the mixture and moisture also influences the flow properties (Beckett, 2009a).

1.8.3 Texture and appearance

Chocolate texture and appearance are key attributes in consumer's choice and acceptability. Although texture perception is a dynamic oral process before and during mastication, individuals also perceive texture through vision, touch an hearing. Chocolate texture can be evaluated by instrumental measurements often rationalized as cheap, efficient and objective replacements or complements for sensory evaluations (Afoakwa, 2010). A general agreement has been reached on the definition of texture which evolved from the efforts of a number of researchers. It states that "texture is the sensory and functional manifestation of the structural, mechanical and surface properties of foods detected through the senses of vision, hearing, touch and kinethetic" (Szczesniak, 2002).
Hardness is a variable that is often used as a criterion of chocolate texture. The hardness of chocolate is mainly influenced by the tempering of the chocolate. Over-tempering leads to increased hardness and stickiness. The particle size, fat and lecithin are inversely correlated to the hardness of tempered chocolate. By manipulating these parameters, the hardness/softness of the product can be improved (Afoakwa, 2010).

Chocolate should meet prior acquired consumer expectations; especially appearance attributes can have significant commercial implications. Appearance attributes include gloss, colour, shape, roughness, surface texture, shininess and translucency. These emerge from complex interactions of incident light, optical characteristics and human perception (Afoakwa, 2010). Appearance is measured frequently as colour. To express the colour of a food product, the CIEL*a*b-system is most often used. This system uses tree spatial coordinates a* (+a* red, -a* green), b* (+b* yellow –b* blue) and L* (lightness) (Völz, 2001). A spectrophotometer can be used to measure the colour. The spectrophotometer measures

the reflected light with or without the specular component, respectively the SCI-mode (specular component included) and SCE-mode (specular light included). If instrumental colour measurements are compared with human observation, the SCE-mode is used because the human eye neglects the specular component (Minolta, 1998).
Colour can also be expressed as lightness (L*), chromaticity (C*) and hue angle (h°). The CIEL*a*b* values are converted to C* and h° by following equations:

$$h° = \arctan\left(\frac{b^*}{a^*}\right)$$

$$C^* = \sqrt{[(a^*)^2 + (b^*)^2]}$$

Increasing particle size and/or increasing fat content results in a decrease of L*, C* and h°. The first effect is explained by the denser packing that results from smaller particles by which the light is more scattered and the chocolate looks more saturated and lighter. The second effect is explained by the fact that when adding more fat a larger crystalline network is present that reduces lightness and saturation (Afoakwa, 2010).

1.8.4 Sensory evaluation

Sensory tests have two main goals. Firstly, obtaining objective information on the sensory properties of a product by the use of a trained panel and secondly, obtaining subjective information about consumers' liking of a product (Kemp et al., 2009).
A trained panel can perform discrimination tests. These tests aim at determining sensory differences between samples. One example of a discrimination test is the duo-trio test. In this test, the assessors taste a reference sample after which they need to taste two blind labeled samples and try to identify the sample which is most similar with the reference sample. Another example is the same-different test by which the assessors get two blind labeled samples and are asked to determine if the samples are the same or different (Kemp et al., 2009).
A second kind of tests that are performed through a trained panel are the descriptive tests that give answers to the following questions: 'In what differ those samples' and 'How much differ those samples'.
The results of sensory analysis can then be related to instrumentally measurable variables as discussed before. Ziegler et al. (2001) indicated that especially the particle size and the yield value influences the perceived mouth feeling. Smaller particle sizes and greater yield values increase the effort to melt and to manipulate the piece of chocolate in the mouth however the intensity of chocolate flavour is not altered.

Chapter 2

Influence of cocoa butter refining on the quality of milk chocolate

Our results suggest that multiple characteristics of chocolate, including sugar, cocoa and the drug-like effects experienced, play a role in the desire to consume chocolate.

Nasser et al., 2011, Physiology and Behaviour, 104, 117-221

2.1 Introduction

2.1.1 Physical refining of edible oils

Fats and oils contain undesired by-products or impurities. These components include free fatty acids, partial acylglycerols, phosphatides, sterols, tocopherols, tocotrienols, hydrocarbons, pigments, vitamins, sterol glycosides, protein fragments, traces of pesticides, dioxins and heavy metals (Gibon et al., 2007). Their content depends on the kind of oil, seed treatment, extraction process and storage conditions. These components can be useful, like tocopherols and tocotrienols which can act as protecting agents against oxidation and possess vitamin E activity. Others are objectionable from a sensory (taste, odour, colour, appearance) or technological point of view (Cmolik and Pokorny, 2000). The most important groups of these minor substances with their deteriorating effects are listed in Table 2.1.

Table 2.1 Minor substances present in crude fats and oils (Cmolik and Pokorny, 2000)

Substance class	Typical representatives	Deteriorating effect
Oxidation products	Volatile aldehydes, ketones, hydrocarbons	Off-flavours
Free fatty acids	Saturated and unsaturated fatty acids	Lower oxidative stability Impaired functional properties
Phospholipids		Lower oxidative stability
Pigments	Chlorophylls, carotenoids, myoglobin	Poor sensory properties
Metal salts	Iron, copper compounds	Lower oxidative stability

To become acceptable for human consumption, most oils must be purified. Mainly a light colour, bland taste and oxidative stability are required. To achieve this, the oils are submitted to several treatments. The objective of oil refining is to remove objectionable minor constituents in the oil with the least possible damage to the acylglycerols and minimal loss of the desirable constituents (Gibon et al., 2007).

Refining is usually done in four steps (Figure 2.1). It can be done by conventional processes, which corresponds to chemical refining or following more recent techniques by using physical refining (Bockisch, 1998). The principal difference between the two routes is how the FFA are removed. If the chemical refining is chosen, the crude raw material is first heated with water or an aqueous solution of phosphoric acid and the sediment, the gums are removed by centrifugation and used separately from the free fatty acids removed in the subsequent alkali refining. FFA are easily removed by washing with a solution of sodium

hydroxide or sodium carbonate. On the other hand, in a physical operation, most FFA are removed in the deodorizing unit. Oil must also be carefully degummed and bleached as the deodorization is a distillation process at high temperature. Following problems could arise when no pretreatment is preformed:

(i) Phosphatides can decompose to products which cannot be removed

(ii) Gossypol (mainly present in cottonseed oil) thermally decomposes to black substances

(iii) Proteins decompose under formation of coloured components and form odoriferous and bad-tasting substances, mainly in conjunction with carbohydrates and (heavy) metals which promote oxidation

The physical refining process can offer important advantages such as a higher oil yield, reduction of the use of chemicals (like phosphoric acid, sulfuric acid and caustic soda), reduction of water and effluent and hence is more environmental friendly. In chemical refining, the soapstock also needs to be split to obtain the FFA. The disadvantage of physical refining is that some oils must be more carefully degummed and bleaching earth consumption is higher. Alkali neutralization also removes a wider range of undesirable products compared to degumming (Bockisch, 1998; Gibon et al., 2007).

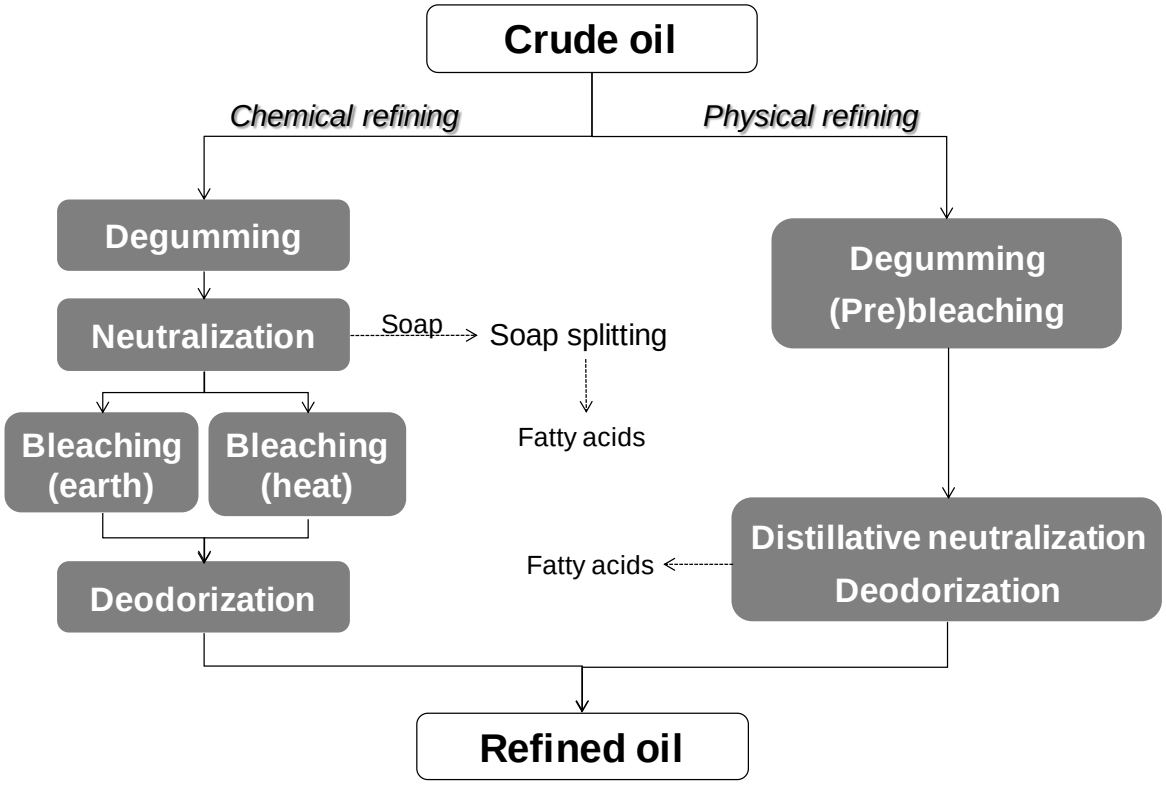


Figure 2.1 Process steps of chemical and physical refining (adjusted after Bockisch,1998)

The final choice between chemical and physical refining will depend on a number of factors: the quality and the acidity of the crude oil, the ability to get rid of the soapstock and local environmental legislation.

2.1.2 Cocoa butter refining

Cocoa butter should contain less than 1.75% free fatty acids (FFA, based on oleic acid) to be in compliance with the EU directive 2000/36/EC (2000) and needs to be free from off-flavours, moulds and rancidity (Calliauw et al., 2008).

The conventional cocoa butter refining process exists of a filtration followed by a batch or continuous deodorization. However, because of the increased industry's demand for different types of cocoa butters (e.g. in terms of colour, degree of neutral flavours, etc), other more flexible refining technologies have become necessary (Vila Ayala et al., 2007). De Greyt et al. (2003) suggested an improved three stage refining process. Firstly, a pretreatment with silica is carried out to adsorb alkaline components, impurities and phosphatides. Secondly, an optional bleaching step is applied to modify the colour, only important for the production of white chocolate and finally packed column stripping or tray deodorization is performed.
In a packed column, which is filled with structured packing material with high surface area (250-350 m^2/m^3), there is an intensive counter-current contact between the oil and the stripping steam resulting in a high stripping efficiency and an overall lower steam consumption. A so-called scrubber is attached to the packed column unit for the condensation of the FFA. Another feature of the packed column is the shorter residence time which allows a higher process temperature (needed for the stripping of the FFA) without unwanted side reactions (interesterification, isomerisation and formation of cyclic and trans fatty acids) (Vila Ayala et al., 2007). Therefore, this technique is very interesting for cocoa butter refining as its unique crystallization properties should be preserved.

2.1.3 Impact of refining on the physicochemical properties

Timms and Stewart (1999) stated that when deodorization is performed at lower temperatures (130 – 180°C) for 10 – 30 minutes, there is no effect on the physical properties of cocoa butter.
Vila Ayala et al. (2007) and Calliauw et al. (2008) did a thorough study of the effect of bleaching and/or packed column steam refining on cocoa butter properties. They concluded that there was no effect on the fatty acid profile, TAG distribution and MAG and DAG

content, even up to temperatures of 260°C. The FFA removal in relation to the oil temperature could be described by a sigmoid function. Furthermore, the bleaching step before steam refining efficiently removed the alkaloids, theobromine and caffeine. Steam refining around 200°C also completely removed these alkaloids. It is known from industrial practice that these alkaloids tend to deposite in the vapour duct between the deodorizer and the vapour scrubber which may give rise to frequent cleaning of the equipment. In the study of the crystallization behaviour of steam refined cocoa butter, it was concluded that the cocoa butter crystallized sooner and faster when FFA were removed.

Physical refining also has an impact on minor components in fats and oils. These may be desirable components such as tocopherols or undesirable ones such as pesticides or polycyclic aromatic hydrocarbons. Tocopherols are an important antioxidant with vitamin E activity. De Greyt et al. (1999) concluded that when applying temperatures between 220°C and 260°C on soy bean oil only 5% of the tocopherols were removed. Tocopherols in rapeseed oil were found to linearly decrease with increasing deodorization temperature. The retention of the individual tocopherols decrease in the same order as their molecular weight, retention α- > retention γ- > retention δ-tocopherol (Cmolik et al., 2008). Timms and Stewart (1999) reported a typical reduction up to 15%, with a typical level in deodorized cocoa butter of 250 ppm.

2.2 Research strategy

As cocoa butter forms the continuous phase of chocolate, it has a major influence on the quality of the final product. Therefore, it is crucial that the cocoa butter has an optimal quality. After pressing, the crude cocoa butter still contains undesired components making it necessary to refine the product. Moreover, due to an increasing demand for chocolate products, the quality of the cocoa butter tends to decrease. As noticed by Vila Ayala et al. (2007), there is an increased industry's demand for different types of cocoa butters (e.g. in terms of colour, degree of neutral flavours, etc). Therefore, the application of a more intense refining process is necessary to obtain a good quality cocoa butter.

In this experimental setup, the method suggested by De Greyt et al. (2003), described in detail in section 2.1.2, was used. The crude cocoa butters were submitted to a silica pretreatment but no bleaching step was performed. As it is important to preserve the unique crystallization properties of cocoa butter, unwanted side-reactions like interesterification, isomerisation should be avoided. Therefore, the short time, high temperature treatment of a packed column steam refining was preferred.

As described in section 2.1.3, the effect of the refining process on the physicochemical properties of cocoa butter has been described by different researchers. However,

information about the impact of different refining conditions on final chocolate quality is limited.

As a result, following key objectives were postulated:

- What are the effects of the silica pretreatment and/or refining step on the physicochemical properties of a crude cocoa butter?
- Does the refining process result in quality changes in milk chocolate?

To answer these questions, three crude cocoa butters, with or without silica pretreatment, were subjected to a steam refining in a packed column at five temperatures: 150°C, 175°C, 200°C, 225°C and 250°C. The impact of the refining treatments on the physicochemical properties was evaluated.

In the next step the refined cocoa butter samples were applied in a milk chocolate formulation. The produced milk chocolates were then evaluated for different quality characteristics such as particle size distribution (PSD), flow behaviour and texture.

2.3 Materials and Methods

2.3.1 Feedstock used for the research

Three un-deodorized crude cocoa butters (CB) were used for the refining experiments. A commercial refined cocoa butter was used as a reference (Table 2.2).

Table 2.2 Overview of the cocoa butters used in this study

CB A	Ivory Coast	Barry Callebaut, Wieze, Belgium
CB B	Malaysia	Nord Cacao, Gravelines, France
CB C	Malaysia	Cargill, Belgium
REF	Unknown	Belcolade, Erembodegem, Belgium

2.3.2 Cocoa butter refining

The physical refining of the different cocoa butters was performed in a pilot plant equipment installed in the R&D center of Desmet Ballestra Group N.V. (Zaventem, Belgium).

2.3.2.1 Silica pretreatment

The silica pretreatment was carried out in a batch reactor according to the following procedure. The CB was first heated to 80°C and 0.6% (v/w) of a 15% (w/w) citric acid solution was added and thoroughly mixed with the CB. After 15 min, 0.5% (w/w) silica was added and agitation continued for another 30 minutes. A vacuum was applied to remove the

water. The silica was separated from the CB in a plate-and-frame filter mounted with a standard filter cloth by pressurizing the reactor with compressed air.

2.3.2.2 Packed column steam refining

The crude and silica pretreated cocoa butters were stripped in a pilot packed column unit (internal column diameter : 7.6 cm, packing height: 2 m) filled with a structured packing with a specific surface area of 250 m^2/m^3. Cocoa butter was pumped at a rate of 13 kg/hr and 1% stripping steam was injected. A top pressure of 3 mbar was applied and pressure drop over the packing was 0.5 – 0.75 mbar. Estimated residence time is approx. 7 min. Cocoa butter temperature was varied between 150°C and 250°C with a 25°C interval.

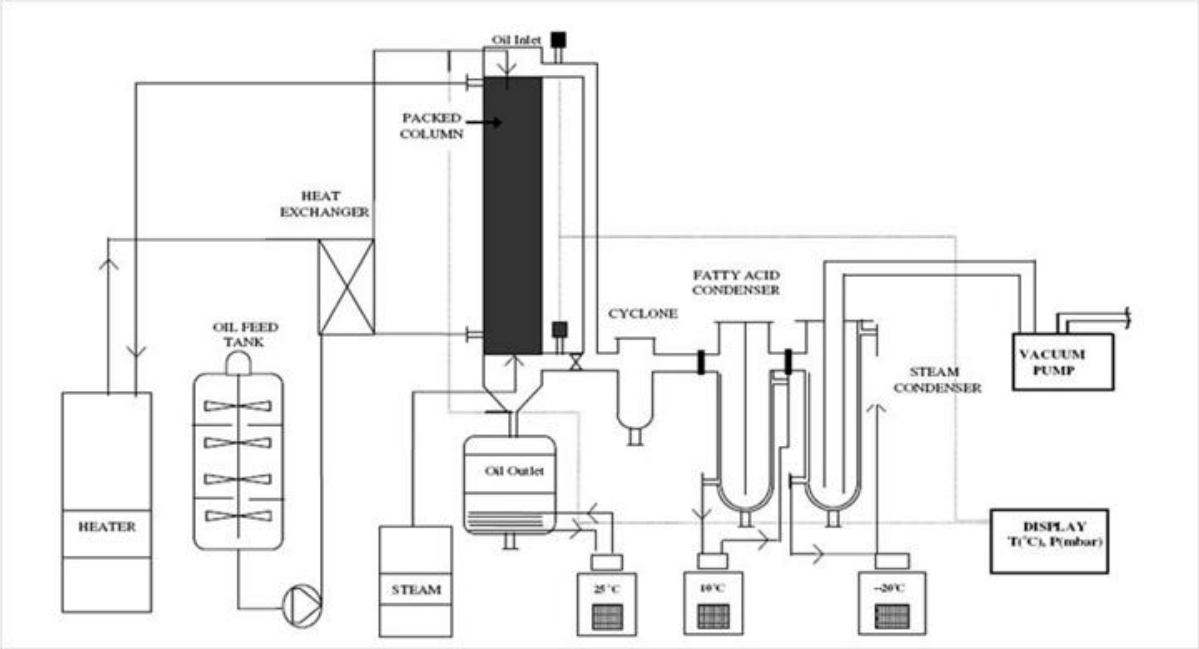


Figure 2.2 Pilot packed column

2.3.3 Chemical analysis

2.3.3.1 Free fatty acids

Free fatty acids (FFA) were determined by using titration according to the AOCS Official Method Ca 5a-40 (Firestone, 1997) and expressed as % oleic acid.

2.3.3.2 Fatty acid methyl ester profile

This AOCS official method Ce 2-66 (Firestone, 1997) was used for preparing methyl esters for further analysis by GC according to the AOCS Official Methods Ce 1-62 (Firestone, 1997).

2.3.3.3 Triacylglycerol distribution

The triacylglycerol (TAG) composition was analyzed by reversed phase HPLC based on the official AOCS method Ce 5b-89 (Firestone, 1997). Minor practical adjustments to the flow rate and mobile phase composition were made. The analysis was performed on a Waters HPLC system (Zellik, Belgium), equipped with two stainless steel Nova-Pak C18 columns (4 µm, 3.9 x 150 mm) (Waters). The mobile phase consisted of an isocratic solvent mixture of acetone and acetonitrile (62.5:37.5) with a flow rate of 1.2 ml/min and the injection volume was 20 µl. Samples were dissolved in methanol/chloroform (1:1, v:v) and a differential refractometer was utilized for the detection. The peaks were identified by comparing their relative retention times with standards. Peak areas were correlated with the quantities of TAGs in the oil or fat sample. They were integrated by using the program Empower Pro with a generic Apex Track method for integration. Peak areas below 4000 area counts (equivalent to approximately 0.04% of the total peak area) were not taken into account.

2.3.3.4 Traces of soaps

The alkalinity was determined as sodium oleate by the titrimetric method described in the official AOCS method Cc 17-75 (Firestone, 1997).

2.3.3.5 Phosphorous (P) and Iron (Fe)

The quantification of phosphorus and iron in oil was done by using inductively coupled plasma optical emission spectroscopy (ICP-OES) as described in the AOCS Official Method Ca 20-99 (Firestone, 1997).

2.3.3.6 Peroxide value

The AOCS Official Method Cd 8b-90 (Firestone, 1997) was used to determine all substances, in terms of milliequivalents of peroxide per 1000 grams of test sample, that oxidize potassium iodide under the conditions of the test. The substances are generally assumed to be peroxides or other similar products of fat oxidation.

2.3.3.7 p-Anisidine value

The p–anisidine value is defined by convention as 100 times the optical density measured at 350 nm in a 1 cm cuvette. The AOCS Official Method Cd 18-90 (Firestone, 1997) method determines the amount of aldehydes (principally 2–alkenals and 2,4–dienals) by reaction in an acetic acid solution of the aldehydic compounds in an oil and the p–anisidine and then measuring the absorbance at 350 nm.

2.3.3.8 Tocopherols

Tocopherols were determined with normal phase HPLC as described by the AOCS official method Ce 8-89 (Firestone, 1997). Cocoa butter (0.3 g) was added in a volumetric flask of 25 ml, filled further with hexane up to 25 ml and shaken thoroughly. Samples were then analyzed on a HP series 1050 chromatograph with FLD (Hewlett Packard, Avondale, PA, USA) using a 0.5 % (v/v) 2-propanol in hexane mobile phase.

2.3.3.9 Theobromine and caffeine

Theobromine and caffeine contents were determined by a reversed phase HP series 1050 chromatograph (Hewlett Packard, Avondale, PA, USA). A HP UV-Visible detector at 272 nm was used as detector. A detailed description of the method is given by Vila Ayala et al. (2007).

2.3.4 Physical analysis

2.3.4.1 Colour

The colour was determined on an automatic Lovibond PFX 880/P provided with a heater to avoid solidification of the cocoa butter. The analysis was performed at 70°C and the colour was expressed in the CIELAB coordinates: the lightness L* (0:black to 100: white), a* (negative values indicate green while positive values indicate red) and b* (negative values indicate blue and positive values indicate yellow).

2.3.4.2 Oil Stability Index (OSI)

The oil stability index (OSI) of the cocoa butter samples was determined according to the Rancimat method as described more in detail in the AOCS Official Method Cd 12b-92 (Firestone, 2009).

2.3.4.3 Differential Scanning Calorimetry (DSC)

The isothermal crystallization behaviour was studied by using differential scanning calorimetery (DSC). DSC is the most widely used of all thermal analysis techniques. It is a technique in which the difference in energy input into a sample and a reference material is measured as function of time or temperature while the sample and the reference are subjected to a controlled time-temperature program. It provides qualitative and quantitative information regarding transitions in materials that involve endothermic and exothermic processes (Foubert, 2003). All analyses were performed on a Q1000 DSC with Refrigerated Cooling System and autosampler System (TA instruments, New Castle, USA). It was calibrated with indium (TA instruments, New Castle, USA), azobenzene (Sigma-Aldrich, Bornem, Belgium) and undecane (Acros organiscs, Geel, Belgium) prior to analysis. Nitrogen was used to purge the system.
A sample (5 – 15 mg) was hermetically filled in alodined aluminum pans. An empty pan was used as a reference. The sample was held isothermal at 65°C for 10 min to ensure a completely liquid state and erase the crystallization memory of the sample. Then, it was cooled at 10°C/min to the desired crystallization temperature (in this case 20°C) and held there for 240 min. Each analysis was executed in triplicate.

The kinetics of the CB crystallization can be described by means of mathematical models. Foubert (2003) has developed a model for fat crystallization based on cocoa butter crystallization kinetics. More details can be found in section 1.6.2. The model was fitted to the integral of the main crystallization peak observed in DSC.

2.3.4.4 Solid fat content

The solid fat content (SFC) is generally measured by pulsed nuclear magnetic resonance (pNMR). A detailed description of this method is given by Vereecken (2010). In this research a Maran ultra pulsed field gradient NMR (Oxford Instruments, Tubney Woods, Abingdon, UK) was used to perform the analyses.

For confectionary fats, a tempering step of 40 hours at 26°C is used to ensure that the cocoa butter is converted to the β polymorph before measurement. The fat was melted and 3.5 ml was placed in NMR tubes (3 replicates) and held at 70°C for 30 min to erase thermal history. Then they were submitted to the tempering treatment of the IUPAC 2.150 serial tempered method. The SFC was determined in the range of 5 – 40°C at 5°C intervals following 60 min incubation in a thermostatic water bath.

2.3.5 Chocolate production

2.3.5.1 Ingredients

Table 2.3 shows the standard recipe of the milk chocolate used in this study. In each production, 3.5 kg of chocolate was prepared. As the goal was to evaluate the effect of CB refining on the chocolate quality, the cocoa mass (CM) was replaced by cocoa powder (CP) and CB.

Table 2.3 Milk chocolate recipe

Ingredient	Amount	Supplier	Specifications
Cocoa butter	25.9 %	See Table 2.2	
Low-fat cocoa powder	9.1 %	Barry Callebaut, Wieze, Belgium	11 – 12 % fat
Whole milk powder	22.5 %	Hochdorf Swiss Milk, Hochdorf, Switzerland	28 % fat; roller dried
Pre-broken sugar	42.1 %	Barry Callebaut, Wieze, Belgium	
Soy lecithin	0.4 %	Barry Callebaut, Wieze, Belgium	

2.3.5.2 Procedure

A flow chart of the milk chocolate production procedure is presented in Figure 2.3.

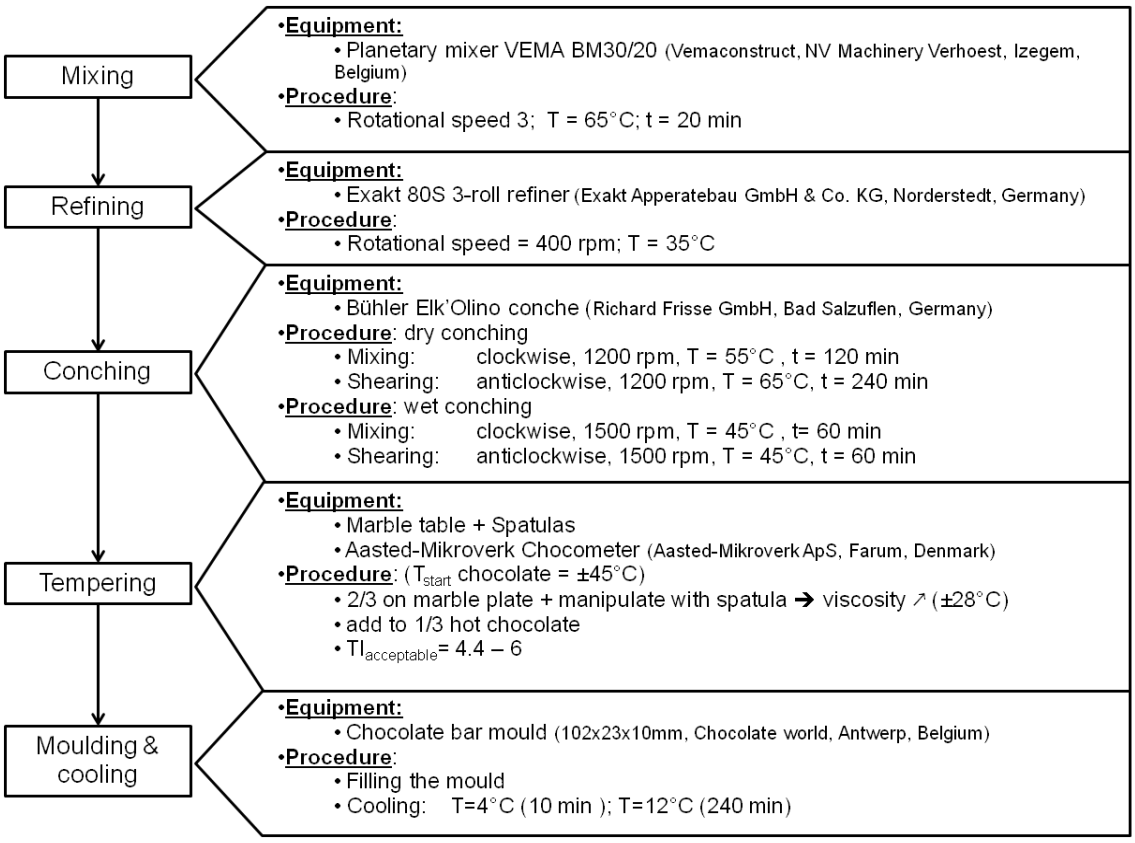


Figure 2.3 Flow chart of the laboratory scale chocolate production process

In the first step, sugar, milk powder, cocoa powder and a part of the cocoa butter were mixed in a planetary mixer Vema BM 30/20 (Vemaconstruct, NV machinery Verhoest, Izegem, Belgium). Cocoa butter was added to obtain a fat content of 27%. In the next step, particle size was reduced by refining on an Exakt 80S 3-roll mill (Exakt Apparatebau GmbH & Co. KG, Norderstedt, Germany). The temperature of the rolls was set at 35°C.
The refined product was then conched in a Bühler Elk'olino conche (Richard Frisse GmbH, Bad Salzuflen, Germany). Conching consisted of two phases: a dry and a liquid conching phase. In the dry phase, the temperature was first set at 55°C while mixing clockwise at 1200 RPM during 120 min. The next step consisted of a shearing phase of 240 min at 65°C with anticlockwise rotation at 1200 RPM. In the liquid phase, the emulsifier, lecithin and the remaining amount of cocoa butter were added to obtain a final fat content of 33.3% and subsequently mixed at 45°C, 1500 RPM for 60 min followed by a shearing step with the same settings.

To temper the liquid chocolate, the temperature was set to 45°C to remove all crystal history. Approximately 2/3 of the chocolate was cooled on a marble plate by manual handling until viscosity was judged as appropriate (temperature ± 28°C). This chocolate was then added to the other third of the remaining one third at 45°C to melt the unstable crystals. To evaluate the correctness of this tempering step, the temper index (TI) and the chocolate tempering unit (CTU) were measured with an Aasted-Mikroverk Chocometer (Aasted-Mikroverk ApS, Farum, Denmark). If a TI of 5 is obtained, a chocolate is considered as well-tempered. The tempered chocolate was moulded in chocolate bars (dimensions bar:102 x 23 x 10 mm; Chocolate World, Antwerp, Belgium) and cooled for 10 min at 4°C followed by 4 hours at 12°C. The bars were subsequently stored at room temperature prior to further analysis.

2.3.6 Quality characteristics of chocolate

2.3.6.1 Particle size distribution

The particle size distribution was measured with a laser diffraction system, the Malvern mastersizer S (Malvern Instruments Ltd., Worcestershire, UK) equipped with a 300 RF lens to measure particles in a range of 0.05 µm up to 900 µm. In the sample preparation 0.5 g of molten chocolate was mixed with 10 ml of isopropanol (VWR, Leuven, Belgium) and put in an oven at 40°C for at least one hour. This was done in triplicate.
In this study, the volume-weighted mean diameter (D[4,3]) and the volume 90% distribution percentile (D[90]) were used. The measurement was repeated five times.

2.3.6.2 Rheology

To measure the flow behaviour of the produced chocolates a TA Instruments AR2000ex (TA Instruments, New Castle, Delaware, USA) with plate-plate geometry was used. The temperature of the bottom plate was set at 40°C. A small amount of chocolate was brought on the plate and a gap of 1000 μm was used. A stepped flow procedure was applied by increasing the shear rate logarithmically from 0.01 s^{-1} to 100 s^{-1} while measuring the shear stress. Every chocolate sample was measured in triplicate. The Casson model was fitted to the shear rate (from 0.1 to 65 s^{-1}) -shear stress data.

2.3.6.3 Texture analysis

A three point bend test was performed on a TA500 Texture Analyzer (Lloyd Instruments Ltd., West Sussex, UK) equipped with a 500N load cell and a plastic cutting probe. The fracturability is the maximum load [N] necessary to fracture a bar (102x23x10 mm) of tempered chocolate. The probe descended at 10 mm/min until the chocolate bar cracked. For every chocolate, ten bars were subjected to the three point bend test.

2.3.6.4 Colour measurement

The colour was measured on a Minolta Model CM-2500D spectrophotometer (Konica Minolta Sensing, Inc., Osaka, Japan). The colour is expressed as a L*-value, an a*-value and a b*-value.

2.3.7 Statistical methods

The measurements of variables that were repeated more than two times are statistically compared using a one-way ANOVA. A Levene test is used to test the hypothesis of equal variances. If this hypothesis is restrained, a Tukey test will detect the significant differences on a 95% significance level. When the variances were not equal, a Dunnett's T3 test was used.

The data were statistically analyzed with SPSS 17.0 software package (SPSS Inc., Chicago, USA). To evaluate significant differences between data, one-way ANOVA (α=0.05) was used. The Shapiro-Wilk test was used to check whether the data were normal distributed. Homogeneity of variance was verified prior to analysis by means of a Levene test. When significant differences were detected, a post-hoc test was executed to evaluate which treatment groups were significantly different. In case of equal variances, Tuckey was used. When variance were significantly different, a Dunnett T3 post-hoc test was used.

Every chocolate was produced in duplicate or triplicate. Intra- and intervariability of the measurements should be taken into account when evaluating the quality parameters of the chocolate, therefore an average value for the parameter was calculated with corrected standard deviation for the repetitions of one kind of chocolate. This was done based on following formula (Foubert, 2003):

$$s = \sqrt{\frac{{s_j}^2}{n_j} + \frac{{s_{j1}}^2 + \cdots + {s_{jn_j}}^2}{{n_j}^2}}$$

With s the corrected standard deviation, s_j is the standard deviation between the different repetitions, n_j is the number of repetitions, $s_{j1,\ldots,nj}$ is the standard deviation within one repetition.

To estimate whether the crystallization parameters differ significantly between groups of experiments an adapted t-test was used as described by Foubert (2003). This adapted t-test takes into account that the calculated parameters of the models are estimates themselves. For finite sample sizes, the null distribution is better approximated by a t-distribution.

2.4 Results and discussion

2.4.1 Characterization of the crude cocoa butters

The most relevant quality characteristics of the three crude cocoa butters considered in this research are listed in Table 2.4. Although CB A has a different origin, the differences between TAG composition are small. All cocoa butters can be classified as hard butters as the amount of SOS-type (S: saturated) TAG is higher than 85%.

The legally established limit for free fatty acids is 1.75% (EU Directive 2000/36/EC). The crude butters clearly exceed this limit which demonstrates the necessity to refine the butters.

The phosphorus content is commonly determined by ashing the sample and measuring the amount of phosphorous spectrophotometrically. In this way no distinction is made between inorganic phosphates and organic phosphatides. However, some conversion factors exist to calculate the amount of phospholipids (Carelli et al., 2002). One ppm phosphorous equals 25 ppm or 0.0025% phospholipids (Foubert, 2003). Consequently, the amount of phosphorous for the cocoa butters in this set up varied between 45 to 63 ppm, corresponding with 0.11 to 0.16% phospholipids respectively. These values are in correspondence with Foubert (2003).

The soap content is expressed as ppm sodium stearate. A cocoa butter of good quality should contain less than 200 ppm of soaps and a cocoa butter has an inferior quality when the value is higher than 4000 ppm. High soap contents may be due to alkalization of the cocoa nibs or mass prior to pressing. If this alkalizing is not carefully performed, saponification can take place (Cros and Bianchi, 1998; Foubert et al., 2003; Pontillon, 1998). Foubert et al. (2004b) reported an average value of 128 ppm in a study of 20 cocoa butters of different origin. Compared to these results, the values for the cocoa butters in this study were quite high, indicating that the butters were extracted from alkalized starting material.

The amount of tocopherols is higher in the commercial butter compared to the crude butters. A value of ~250 ppm is in correspondence with the observations of Timms and Stewart (1999). Four types of tocopherols were detected: α-tocopherol, β-tocopherol, γ-tocopherol and δ-tocopherol, with γ-tocopherol and α-tocopherol comprising the majority of approximately 85% and 10% respectively in all samples.

The presence of these tocopherols and the favorable fatty acid distribution (low amounts of C18:2 (<3%) and traces of C18:3), provides that cocoa butter has a good resistance to oxidation at moderate temperatures (<80°C) in the absence of light (Pontillon, 1998). This was reflected in the oil stability index (OSI) which showed high values (> 32 hours). The present status of oxidation is measured by the peroxide and p-anisidine value (Coppin and Pike, 2001). The peroxide values of the crude butters are higher than the reference butter

although the latter has a higher p-anisidine value, indicating that secondary oxidation took place.

Table 2.4 Acylglycerol distribution and chemical quality parameters of the crude cocoa butters (A, B and C) compared to a commercial cocoa butter (Ref)

	CB A	CB B	CB C	CB Ref
Origin	Ivory Coast	Malaysia	Malaysia	unknown
Distribution (%w/w)				
MAG/DAG	2.32	2.27	1.50	1.74
LOO	0.41	0.55	0.52	0.38
PLO	0.56	0.82	0.57	0.53
PLP	1.87	1.89	2.14	1.97
OOO	0.28	0.29	0.19	0.67
POO	2.7	2.89	2.02	3.07
POP/PLSt	18.36	17.26	18.08	18.31
StOO/StLSt	2.86	3.30	2.47	2.83
POSt	39.31	38.66	40.24	38.69
PPS	0.30	0.28	0.37	0.46
StOSt	27.16	27.27	27.25	27.29
PStSt	0.46	0.72	0.85	0.48
StOA	1.60	1.87	1.36	2.03
SSS	0.33	0.37	0.28	0.25
FFA content (%)	3.05±0.02	2.14±0.01	2.31±0.01	1.54±0.01
Phosporous (ppm)	44.97±0.10	63.52±0.31	48.15±0.21	58.55±0.24
Tocopherols (ppm)	244.21	264.54	224.48	187.97
Theobromine (ppm)	61.75	75.71	73.71	51.43
Caffeine (ppm)	293.17	437.98	389.52	197.51
Alkalinity (ppm sodium stearate)	183±7	392±13	250±15	306±29
Peroxide (meq O_2/kg oil)	2.87±0.10	4.31±0.10	1.54±0.18	0.85±0.04
p-anisidine	0.82±0.05	0.60±0.06	0.57±0.01	2.09±0.16
OSI (hours)	45.68±1.38	32.43±1.53	34.38±1.60	36.43±1.17

2.4.2 Influence of silica pretreatment and/or packed column steam refining on cocoa butter properties

2.4.2.1 Experimental design

The refining experiment was executed for the three different crude CBs. Every CB was thoroughly mixed and split in two parts. One part of the butter was treated with 0.5% silica.

The non-pretreated and pretreated CBs were then processed through the packed column at temperatures ranging from 150°C to 250°C with a 25°C temperature interval. In this way every crude CB resulted in eleven cocoa butter samples with different refining history as illustrated in Figure 2.4.

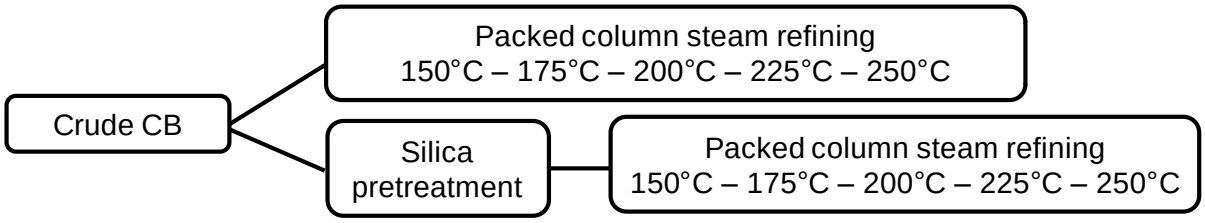


Figure 2.4 Overview experimental design

In this part of the study, the aim was to determine the effect of the physical refining on physicochemical properties of the CBs. First the major components, i.e. TAG and FA-profile, are discussed and then the minor components, i.e. FFA, phosphorous, alkalinity, tocopherols, theobromine, caffeine, MAG, DAG and sterols are evaluated. Thereafter physical parameters, like colour and the oxidative stability, i.e. peroxide value, p-anisidine value and oil stability index are discussed. The influence on the crystallization and melting behaviour was also considered. In the discussion below, the results of CB B were used as a representative example. If differences were observed for the other two crude cocoa butters, this will be notified in the discussion.

2.4.2.2 Fatty acid (FA) profile and acylglycerol composition

Silica pretreatment, nor steam refining had an effect on the FA profile and TAG distribution of the cocoa butters. Also the amount of MAG, DAG and sterols did not significantly change during the silica treatment nor during the steam refining. This indicated that no *cis-trans* isomerisation occurred, nor that fatty acids were exchanged between the TAG. So even at the most severe conditions, the short residence time of the butter in the packed column was sufficiently effective to circumvent unwanted side-effects involving the TAG (Calliauw et al., 2008).

2.4.2.3 Removal of minor components

- *Removal of free fatty acids*

In a physical refining, FFA are removed during the steam refining. Together with other volatile components they are stripped and condensed in the scrubber. Figure 2.5 shows the FFA-content as a function of the packed column temperature for the crude CB with and without the silica pretreatment. To show the crude CB and the CB with silica pretreatment (Sil) without packed column steam refining on the same figure as the packed column steam refined samples, the first two are coded on the temperature axis as NA (not applicable). The silica treatment slightly increased the FFA (not significantly). A clear effect of steam refining on FFA content was found: the higher the applied temperature, the lower the FFA content. Independent of the pretreatment, the FFA content dropped below the legal limit of 1.75% at T ≥ 200°C. The curve of FFA removal as a function of temperature showed a typical sigmoid shape as in accordance with the findings of Vila Ayala et al. (2007). At temperatures ≥ 225°C almost all the FFA were removed.

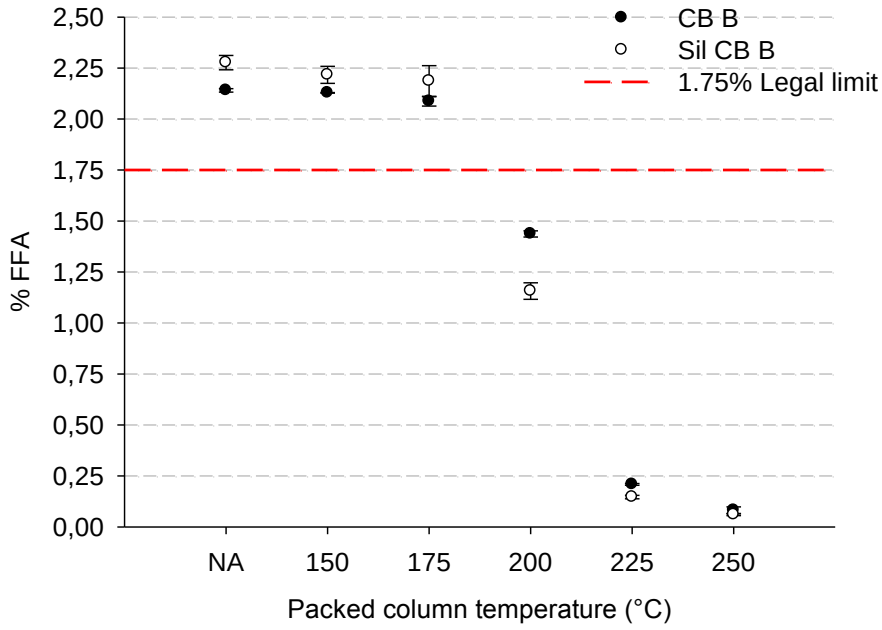


Figure 2.5 FFA-removal as function of temperature during packed column steam refining (NA: not applicable, Sil: silica pretreatment)

- *Other minor components*

In Table 2.5 the results for the other minor components (trace elements, soaps, tocopherols, alkaloids) are listed as function of the refining treatment.

Phosphorous is mainly present in CB as phospholipids. The silica treatment acted as a dry degumming process since the silica pretreatment reduced the phosphorous content with

more than 85%. A similar reduction was observed for *iron*. As stated by Gibon et al. (2007) a citric acid solution is more effective compared to phosphoric acid for the removal of iron. The steam refining at the different temperatures did not significantly influence the amounts of these trace elements.
The silica treatment reduced the amount of soaps or the *alkalinity* to an undetectable level. There was no clear trend of temperature during the packed column steam refining but overall a decreasing trend, varying between 7 to 30%, was observed.

A silica treatment as well as a steam refining decreased the *theobromine* and the *caffeine* content. The silica treatment removed more than 50% of the theobromine but was less efficient in absorbing the caffeine as still 76% of the original amount was present. The silica pretreatment was also less efficient compared to a bleaching pretreatment as Vila Ayala et al. (2007) observed a 90 and 95% decrease of theobromine and caffeine respectively due to bleaching. When the silica treatment was combined with a steam refining, theobromine and caffeine were no longer detected when temperatures ≥ 200°C were applied. Without the silica pretreatment, theobromine and caffeine were completely removed at temperatures >225°C. The removal of the alkaloids during this steam refining can cause problems as these deposit on the vapour scrubbers, decreasing its efficiency. A pretreatment is therefore of interest as the amount of alkaloids is decreased before entering the packed column. It can be concluded that a bleaching treatment is more efficient compared to a silica pretreatment to remove the alkaloids.

As part of the unsaponifiable fraction, *tocopherols* were analyzed. The silica treatment resulted in a small reduction of the amount of tocopherols. During the steam refining, maximum 30% is removed at the highest packed column temperature. CB A and CB C showed a reduction of 20-25% at temperatures >200°C. Pontillon (1998) reported that tocopherols were destroyed by deodorization due to the high temperatures (140 – 160°C). Timms and Stewart (1999) reported a reduction up to 15% in the temperature range 130 – 180°C during batch steam refining.

Table 2.5 Overview table of minor components (trace elements, soaps, unsaponifiable matter, alkaloids) of the cocoa butter as function of refining treatment [*,]**

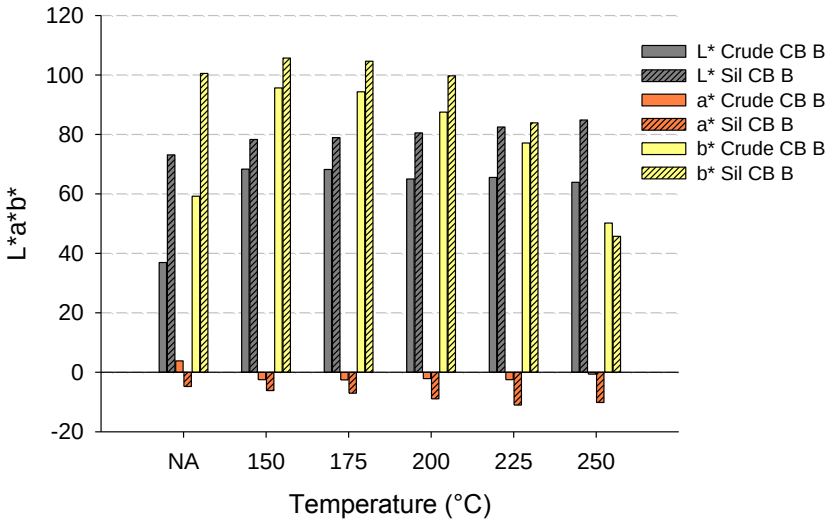
	Trace elements		Alkalinity	Tocopherols	Alkaloids	
	P (ppm)	Fe (ppm)	(ppm Na oleate)	(ppm)	Theobromine (ppm)	Caffeine (ppm)
Crude	63.52±0.30	3.21±0.01	392±13[a]	264.54	75.71	437.96
150°C	62.87±0.08	3.24±0.01	364±30[a]	227.11	75.73	420.51
175°C	63.38±0.19	3.21±0.03	272±24[b]	203.34	65.78	291.41
200°C	64.52±0.25	3.29±0.01	295±14[b]	213.28	27.51	86.01
225°C	64.84±0.18	3.32±0.01	294±22[b]	208.89	/	/
250°C	65.30±0.20	3.40±0.03	306±17[b]	177.96	/	/
Sil	8.66±0.06	0.24±0.00	/	246.55	35.17	331.36
Sil 150°C	8.91±0.05	0.25±0.01	/	223.98	33.31	307.30
Sil 175°C	8.81±0.09	0.24±0.01	/	228.66	29.68	241.16
Sil 200°C	8.78±0.10	0.25±0.01	/	230.83	/	/
Sil 225°C	9.41±0.13	0.35±0.02	/	223.93	/	/
Sil 250°C	9.24±0.06	0.31±0.01	/	207.75	/	/

* / Below detection limit
**Values indicated with the same letters are not significantly different (α=0.05)

2.4.2.4 Colour

Figure 2.6a gives an overview of the colour changes that took place during silica pretreatment and steam refining, expressed in the CIE L*a*b* colour scale. The silica pretreatment on the crude cocoa butter resulted in a lighter i.e. bleached and a more pronounced yellow butter as the L*- and b*-values almost doubled. The positive a* of the crude cocoa butter became negative, indicating a change to the green side of the colour scale. The packed column steam refining temperature also affected the colour. Steam refining has a rather small influence on L*. For both silica treated and untreated cocoa butter samples, b* first increased at 150°C to further decrease with increasing packed column temperature. As colour is determined by the combination of the three individual CIEL*a*b* values, the corresponding colour evolution is presented in Figure 2.6b. The crude cocoa butter clearly shows the darkest colour. The silica pretreatment had undoubtedly a bleaching effect. The steam refining also changed the colour and this was more pronounced at higher temperatures. Thus, the applied refining conditions results in end products with specific colour characteristics.

(a)



(b)

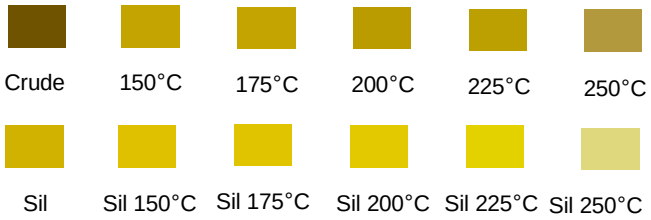


Figure 2.6 (a) L*a*b* as a function of packed column temperature for crude and silica pretreated cocoa butter; (b) colour as a function of packed column temperature for crude and silica pretreated cocoa butter

2.4.2.5 Oxidative properties

To appraise the quality of refined samples, it is also important to investigate the oxidative status. The complex oxidation process can be summarized into two phases: first fatty acids react with oxygen to produce peroxides as odorless compounds (primary oxidation products, measured as peroxide value), which are then degraded into volatile aldehydes and other compounds, responsible for the rancid odour and flavour, and also a non-volatile fraction (secondary oxidation products, measured as p-anisidine value) (Van Hoed et al., 2009).

The silica pretreatment caused a reduction of the primary oxidation products (Figure 2.7a). The p-anisidine value didn't significantly increase ($p>0.05$) during this treatment (Figure 2.7b).

When the temperature of the packed column increased the amount of primary oxidation products decreased. This can be due to the accelerated transformation of primary oxidation products to secondary oxidation products, especially at column temperatures $\geq$ 200°C. Indeed, it can be seen that the secondary oxidation products increased with a higher packed column temperature.

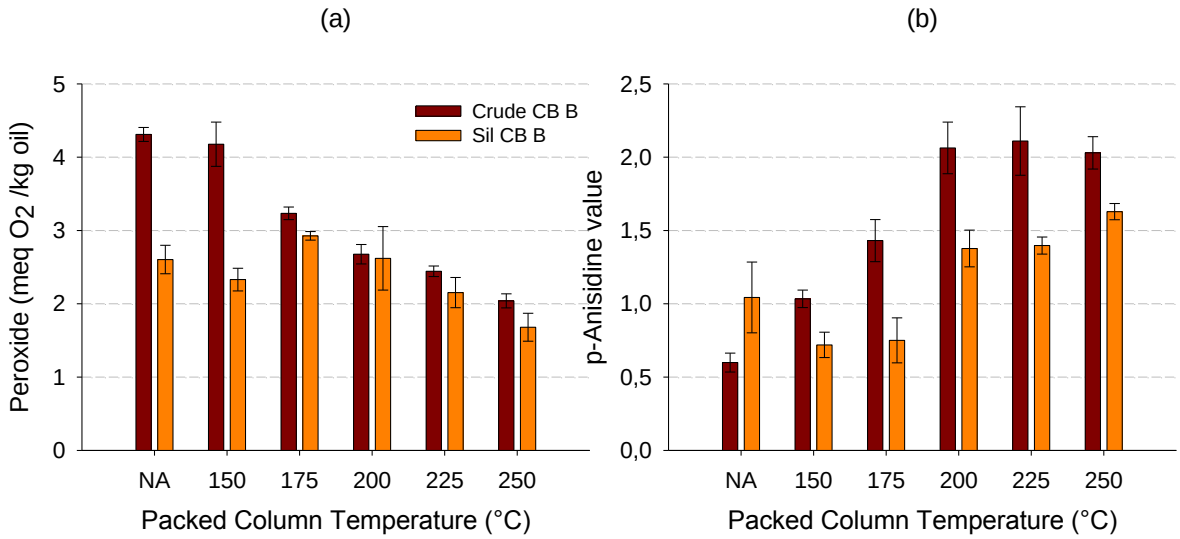


Figure 2.7 Peroxide value (a) and p-anisidine value (b) as a function of packed column temperature for crude and silica pretreated cocoa butter

While the previous parameters give an idea of the actual oxidative status, the oil stability index (OSI) gives information about the shelf life of the cocoa butter. The values are presented in Figure 2.8. The silica pretreatment almost doubled the OSI. A similar trend was observed for the other CBs. An explanation can be found in the removal of the pro-oxidant iron during this pretreatment. There was a tendency that steam refining leads to slightly lower OSI values at temperatures ≥ 200°C. This finding was more clear for CB A and CB C (Annex I-A). A possible explanation of this decrease could be found in the simultaneous removal of the antioxidative tocopherols at these higher temperatures.

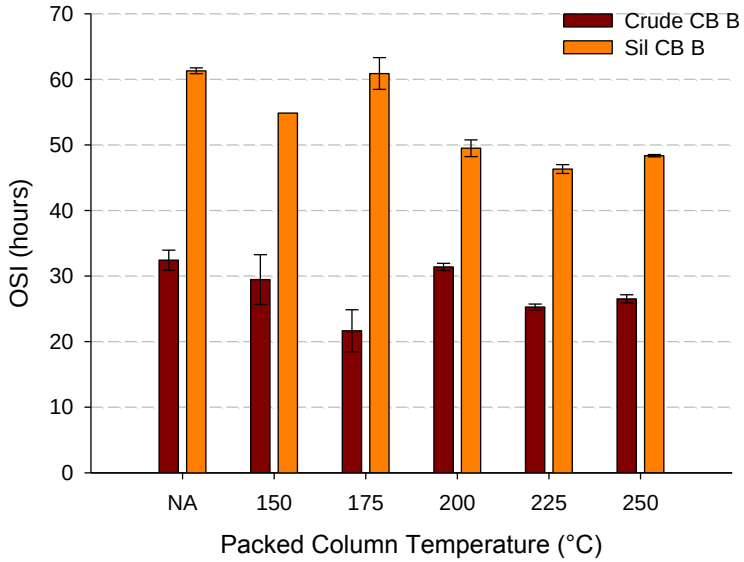


Figure 2.8 Oil stability index (OSI) as a function of packed column temperature for crude and silica pretreated cocoa butter

2.4.2.6 Crystallization behaviour

2.4.2.6.1 Impact on β' kinetics

Foubert et al. (2004b) observed that minor components have an influence on the isothermal crystallization of cocoa butter. Therefore, isothermal crystallization experiments were carried out to define the crystallization behaviour: cocoa butter samples were crystallized at 20°C for 240 minutes. Foubert (2003) has shown that in similar conditions crystallization occurs in two steps: initial formation of α-crystals which quickly transform to the β' polymorph. The β'-crystallization kinetics can be described by the four parameter Foubert-Model (Foubert et al., 2002) in which:

- a_f (J/g): amount of heat released after reaching equilibrium conditions
- K(h^{-1}): rate constant
- n (-): order of the reversed reaction
- t_{ind}(h): time needed to achieve 1% of crystallization

Vila Ayala et al. (2007) concluded that n didn't attribute to the mechanistic interpretation of the kinetics. Besides, this parameter also seems to be correlated with parameter K, therefore the value of n was fixed at six to determine the changes in K (Sichien, 2007).

The main influence of the silica pretreatment was the removal of phospholipids and soaps. Foubert (2003) observed a significant effect of the amount of soaps on the rate constant K: the higher the amount of soaps, the lower K. Phosphorous, together with diacylglycerols and free fatty acids had a positive influence on the induction time. A silica pretreatment of CB B resulted indeed in a significant increase of the rate constant K (crude CB: 2.77±0.16 h^{-1} vs. Sil CB: 3.17±0.06 h^{-1}) while t_{ind} on the other hand didn't significantly increase (crude CB: 0.49±0.06 h vs. Sil CB: 0.56±0.04 h). Parameter a was not significantly influenced. The trends of CB B were compared with results of the other crude butters A and C but no clear conclusions could be drawn as the observations were inconsistent. So the effect of the silica pretreatment on the isothermal crystallization behaviour was not apparent.

As demonstrated in section 2.4.2.3, the main effect of the packed column steam refining was the removal of FFA. Vila Ayala (2007) demonstrated that FFA reduction is a good indicator for the impact of steam refining on crystallization. Therefore results of the Foubert-model parameters were plotted as function of the amount of FFA in Figure 2.9. It was not possible to fit a regression line with an acceptable R^2 (R^2>0.9) to the data points.

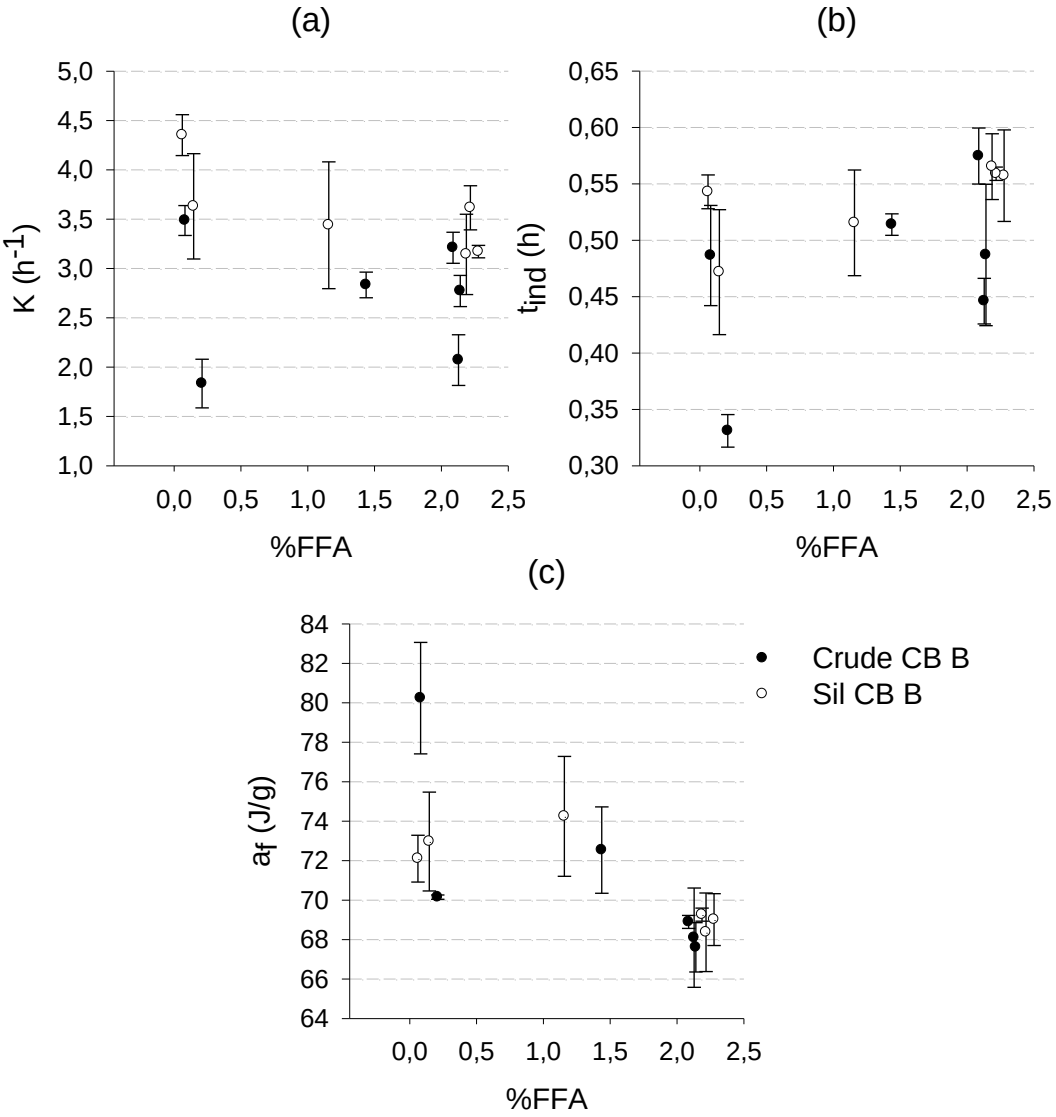


Figure 2.9 Foubert-model parameters K (a), t_{ind} (b) and a (c) as function of the residual FFA for the refined samples of CB B

Combined with the results of the CB A and CB C (Annex I-B and C) following trends were observed:

- The rate constant K had the tendency to increase with increasing steam refining temperature, or K seems to be inversely related with the amount of FFA. Thus the FFA slow down the β' crystallization step.
- The t_{ind} showed a decreasing trend as a function of packed column temperature and consequently as a function of the amount of FFA.
- The amount of heat released during β'-crystallization tended to increase with FFA removal.

Similar to the findings of Vila Ayala et al. (2007) and Calliauw et al. (2008) these results indicate that an increasing temperature in packed column steam refining resulted in a cocoa butter which crystallizes sooner and faster.

2.4.2.6.2 Impact on solid fat profiles

The equilibrium solid fat content of a cocoa butter can be measured by pNMR, after a tempering procedure of 40 hours at 26°C. The SFC profile is an essential quality criterion toward chocolate manufacturing (Calliauw et al., 2008). The typical SFC curve of cocoa butter can be divided into different zones related to the performance of the cocoa butter: a zone up to 25°C is related to the hardness. The zone between 20 – 27.5°C gives an indication of the melting resistance. In the steepest zone (27 – 32.5°C), cocoa butter melts rapidly, releasing flavours, giving a pleasant fluidity in the mouth and a slight cooling sensation. No substantial solid fat may be present at temperature above 35°C, meaning that cocoa butter is completely melted at body temperature and thus no waxy mouth feeling persists after eating (Foubert, 2003; Pontillon, 1998; Talbot, 2009b).

Table 2.6 presents the results of the measurements at 20°C, 25°C and 30°C. Comparing the SFC results of the crude with the silica pretreated CB (not steam refined) it was shown that the silica pretreatment resulted in slightly lower SFC values. The same trend was observed for the crude vs. the silica pretreated samples at the same packed column temperature. At $SFC_{20°C}$ however, the silica pretreated samples, steam refined at 200°C or more showed a significantly higher SFC value. It can be clearly deduced that the difference between the samples with and without silica pretreatment was the most distinct for $SFC_{30°C}$. The SFC of the silica pretreated samples was significantly lower over the whole temperature range. This difference could be explained by the removal of minor components like phospholipids and soaps during silica pretreatment. It has been postulated that minor components, such as glycolipids, phospholipids and saturated TAG, may serve as seed crystals and promote the crystallization of cocoa butter (Chaiseri and Dimick, 1995).

The applied packed column temperature had a clear effect on the SFC: the SFC increased significantly when temperatures higher than 200°C were applied. This indicated again the correlation with the FFA removal as around 200°C, most of the FFA were removed. Therefore, SFC at 20°C, 25°C and 30°C were plotted as function of %FFA in Figure 2.10. The greatest effect of FFA removal was observed at 30°C: the difference between the crude and the steam refined at 250°C was more than 8%; for the silica pretreated vs. its counterpart at 250°C the difference was around 5%. Calliauw et al. (2008) and Vila Ayala et al. (2007) found a positive linear correlation between FFA removal and SFC, indicating that FFA removal has a positive effect on the formation of β-crystals in cocoa butter. These

authors postulated that the degree of crystallization of the TAG is linearly affected by the FFA content. Therefore a regression was performed on the data in Figure 2.10. For the cocoa butter without pretreatment only $SFC_{25°C}$ gave an acceptable linear fit (R^2= 0.9). For the silica pretreated butter, the FFA level explained respectively 94%, 90% and 98% of the variation of SFC at 20°C, 25°C and 30°C.

It can be excluded that the observed changes in SFC are not due to intramolecular changes as one would expect lower SFC values in the range of 20-30°C and significant tailing at 35°C as a result of high-melting trisaturate TAG (Calliauw et al., 2008). Indeed, the SFC showed an increasing trend and no residual SFC was observed at 35°C.

Table 2.6 Equilibrium solid fat content (SFC) of cocoa butter B at 20°C, 25°C and 30°C*

SFC @	20°C	25°C	30°C
Crude	77.13±0.29[a]	68.27±1.19[a]	43.40±0.57[a]
150°C	76.23±0.59[a]	68.63±0.31[a]	48.17±0.58[b]
175°C	76.47±0.35[a]	68.40±0.17[a]	48.23±0.40[b]
200°C	76.57±0.32[a]	69.23±0.67[a]	47.20±0.69[b]
225°C	77.03±0.90[a]	71.73±0.40[b]	52.60±0.78[c]
250°C	77.07±0.23[a]	70.80±0.95[b]	51.10±1.10[c]
Sil	76.37±0.96[a]	67.35±0.45[a]	41.90±0.61[a]
Sil 150°C	76.87±0.47[a]	67.97±0.38[a]	42.03±0.67[a]
Sil 175°C	76.67±0.15[a]	67.53±0.06[a]	41.73±1.70[a]
Sil 200°C	78.30±0.53b	69.67±0.64[b]	44.07±0.61[b]
Sil 225°C	78.80±0.35[b]	70.67±0.75[b]	46.73±0.60[b]
Sil 250°C	78.77±0.35[b]	69.83±0.64[b]	46.10±0.53[b]

* Values indicated with same letters are not significantly different (α=0.05)

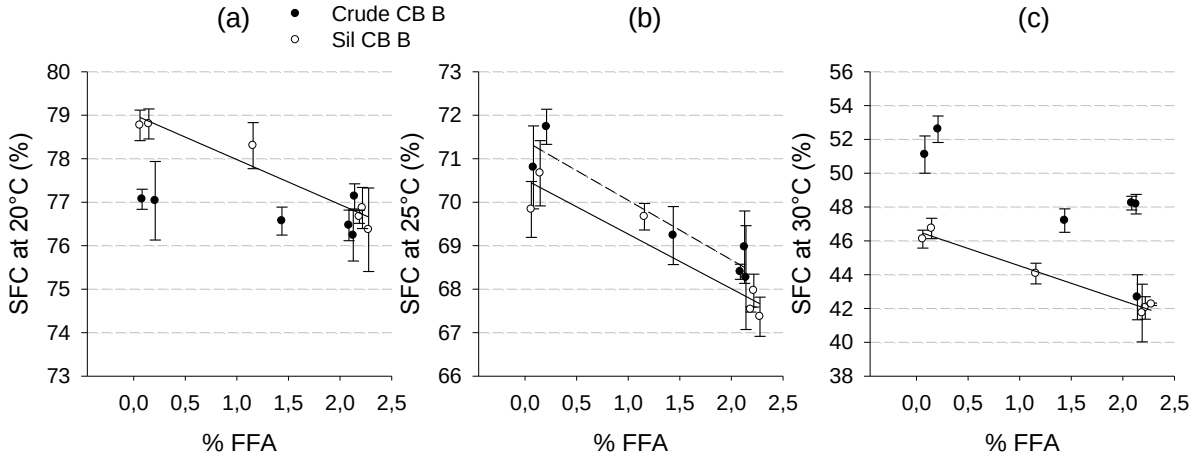


Figure 2.10 Equilibrium solid fat content (SFC) of cocoa butter B at 20°C (a), 25°C (b) and 30°C (c), linear regression lines for which R^2 >0.9 are indicated

Thus it can be concluded that besides the effect on crystallization kinetics, the packed column refining also resulted in cocoa butters with a higher solid fat content (most pronounced at 30°C) at equilibrium conditions.

2.4.2.7 Summary

To summarize, Table 2.7 gives an overview of the main changes caused by the silica treatment and the temperature applied during packed column steam refining.
It can be concluded that the physical refining process alters the chemical composition, with implication on the physical properties. The silica pretreatment clearly results in an improved oxidative stability of the cocoa butter. The major impact of packed column steam refining, FFA removal seemed to be related with changes in cocoa butter crystallization properties. The question remains whether this has an impact on milk chocolate properties.

Table 2.7 Overview of the effects of silica treatment and the temperature applied during packed column steam refining as function of the applied temperature

	Silica pretreatment	**Steam refining (increasing T)**
FFA (%)	No effect	Sigmoid decrease
Phosphorous (ppm)	>85% reduction	No influence
Iron (ppm)	>90% reduction	No influence
Alkalinity (ppm)	Complete removal	Limited influence
Theobromine (ppm)	>50% decrease	Complete removal T≥ 200°C
Caffeine (ppm)	>25% decrease	Complete removal T≥ 200°C
Tocopherols (ppm)	Limited influence	~30% decrease
Colour	Bleaching effect, more yellow	Decrease b*
OSI (hours)	Doubling	Limited influence
Peroxide value (meq O_2/kg vet)	Small decrease	Decrease
p-AV	No clear effect	Increase
Isothermal crystallization at 20°C	No clear influence	Increase K, decrease of t_{ind}
SFC	Decrease	Increase

2.4.3 Influence of cocoa butter refining on the quality of milk chocolate

2.4.3.1 Experimental design

In the next step of the study, the aim was to evaluate the effect of the refining process on the quality of milk chocolate. For that purpose, the milk chocolate production process was first optimized. As the conching phase is very important in both flavour and structural development of chocolate, the optimization mainly focused on variation of the dry conching step. For that purpose, the mixing step during dry conching was kept constant at 2 hours but the duration of the shearing step was varied (4 hours, 6 hours, 10 hours, 14 hours and 24 hours). The variation of this shearing step, had an influence on the flow behaviour as illustrated in Annex I-D. Up to 10 hours dry conching, yield stress nor viscosity changed. Longer dry conching, significantly increased the yield stress, viscosity decreased after 26 hours dry conching. This long conching period also significantly reduced the particle size distribution (PSD). A consumer panel (n= 35) was used to evaluate the different chocolates for different descriptors as well as total preference. As from a practical point of view these long conching times were not desired, only conching time 6h, 8h and 10h were evaluated. No significant differences were found between the descriptors however when considering the total preference, there was a significant difference between 6h and 10h but no difference between 8h and 10h. Based on these findings it was decided to select a dry conching phase of 8 hours: 2 hours of mixing combined with 6 hours shear. A more detailed production process is given is section 2.3.5.

This optimized procedure was used to produce milk chocolates with all the refined samples of CB B. To verify the results, milk chocolate was also produced with the extremes from CB A and CB C. A schematic overview of all the produced chocolates is presented in Table 2.8.

Table 2.8 Chocolate productions

	Silica	**N.A.**	**150°C**	**175°C**	**200°C**	**225°C**	**250°C**
CB A	0%	X			X	X	X
CB A	0.5%	X			X	X	X
CB B	0%	X	X	X	X	X	X
CB B	0.5%	X	X	X	X	X	X
CB C	0%	X					X
CB C	0.5%	X					X

Every chocolate was made in double. To compare the produced milk chocolates, following quality parameters were evaluated: PSD, flow behaviour, colour and texture. Intra- and intervariability of the measurements should be taken into account when evaluating the

quality parameters of the chocolate, therefore an average value for the parameter was calculated with corrected standard deviation as described in section 2.3.7.

2.4.3.2 Particle size distribution

The particle size distribution (PSD) was measured by laser diffraction. PSD, a key determinant of the flow behaviour, affects viscosity as well as texture. The largest particles are important for the mouthfeel with respect to grittiness, but the smaller ones are more important with respect to chocolate flow properties (Afoakwa et al., 2008c). Two parameters were considered: the cumulative fraction D[90] (indicates the size of particles below which 90% of the particles lies) and the volume weighted mean diameter D[4,3]. Table 2.9 shows the D[90] value for the different chocolates produced with the crude and refined cocoa butters. The D[90] was on average 25.5 μm and the mean particle diameter was around 11 μm (results not shown), which were acceptable values as it is necessary to grind all sugar and other solid particles below about 30 μm to avoid the chocolate tasting gritty (Beckett, 2009a). No significant differences were observed between the different samples, although the chocolates produced with CB B, steam refined at 225°C and 250°C has a significant higher D[90] and D[4,3]. This was not observed for the other butters so these values can be considered as outliers.

Table 2.9 D[90] (μm) of chocolate produced with crude and refined cocoa butters

	Silica	None	150°C	175°C	200°C	225°C	250°C
CB A	0%	25.5 ± 0.8					24.6 ± 2.4
CB A	0.5%	26.6 ± 0.5					24.8 ± 1.5
CB B	0%	26.6 ± 1.2	24.9 ± 1.8	25.9 ± 0.4	26.0 ± 1.9	28.0 ± 0.5	27.2 ± 1.1
CB B	0.5%	25.6 ± 2.0	24.0 ± 0.8	24.0 ± 1.8	26.6 ± 1.3	25.8 ± 0.3	23.8 ± 3.0
CB C	0%	26.1 ± 3.1					26.8 ± 0.8
CB C	0.5%	24.1 ± 1.0					26.5 ± 2.4

2.4.3.3 Flow behaviour

Chocolate is an example of a solid suspension, namely a polydisperse suspension of sugar, cocoa and/or milk solids in a Newtonian fluid (fat phase). Therefore chocolate is known to show Non-Newtonian behaviour (Beckett, 2009a). Viscosity is determined by composition, processing parameters and particles size distribution as described more in detail in section 1.8.2. The Casson model was fitted to the flow curves to obtain the Casson yield stress (σ_{Ca}) and viscosity (η_{CA}).

2.4.3.3.1 Effect of cocoa mass replacement on flow behaviour

In this experimental setup it was chosen to replace the cocoa mass by cocoa powder and cocoa butter to observe the maximum effect of the refined cocoa butter. This involved a higher amount of free fat compared to a standard milk chocolate recipe with cocoa mass: namely 25.9 % vs. 19% (Table 2.10). However, this is theoretically, as one should keep in mind that during conching the trapped fat of the cocoa mass is released. To investigate the effect of this replacement on the flow behaviour, two chocolates where produced accordingly with the commercial cocoa butter (Ref., Table 2.4). The results of the rheological measurements are presented in Table 2.10. It is clear that the chocolate containing the cocoa mass has a significantly higher Casson yield stress and viscosity. This may indicate that even after the conching, there is still a difference in the amount of free fat, indicating that not all the trapped fat is released. Free fat enables the chocolate to flow (Beckett, 2009a). More free fat resulted in a higher distance between the solid particles and so the viscosity and yield value is lower for choc 2.

Table 2.10 Amount of free fat, Casson yield stress (σ_{Ca}) and Casson viscosity (η_{CA}) for two different chocolate recipes. CM: cocoa mass; WMP: whole milk powder; CP: cocoa powder; CB: ref. cocoa butter

	% Free fat	σ_{Ca} (Pa)	η_{CA} (Pa.s)
Choc 1: CM + WMP	19.0	12.66 ± 0.21	2.37 ± 0.11
Choc 2: CP + CB + WMP	25.9	7.97 ± 0.18	1.53 ± 0.02

2.4.3.3.2 Flow behaviour of the produced milk chocolates

The flow curves of the different milk chocolates produced with the refined CB samples were analyzed by fitting the Casson model. The results for Casson yield stress and Casson viscosity as function of pack column temperature and FFA removal for CB B are presented in composed Figure 2.11 and those for CB A in composed Figure 2.12. The rheological parameters of CB C are listed in Table 2.11. The results will be discussed in the next paragraph.

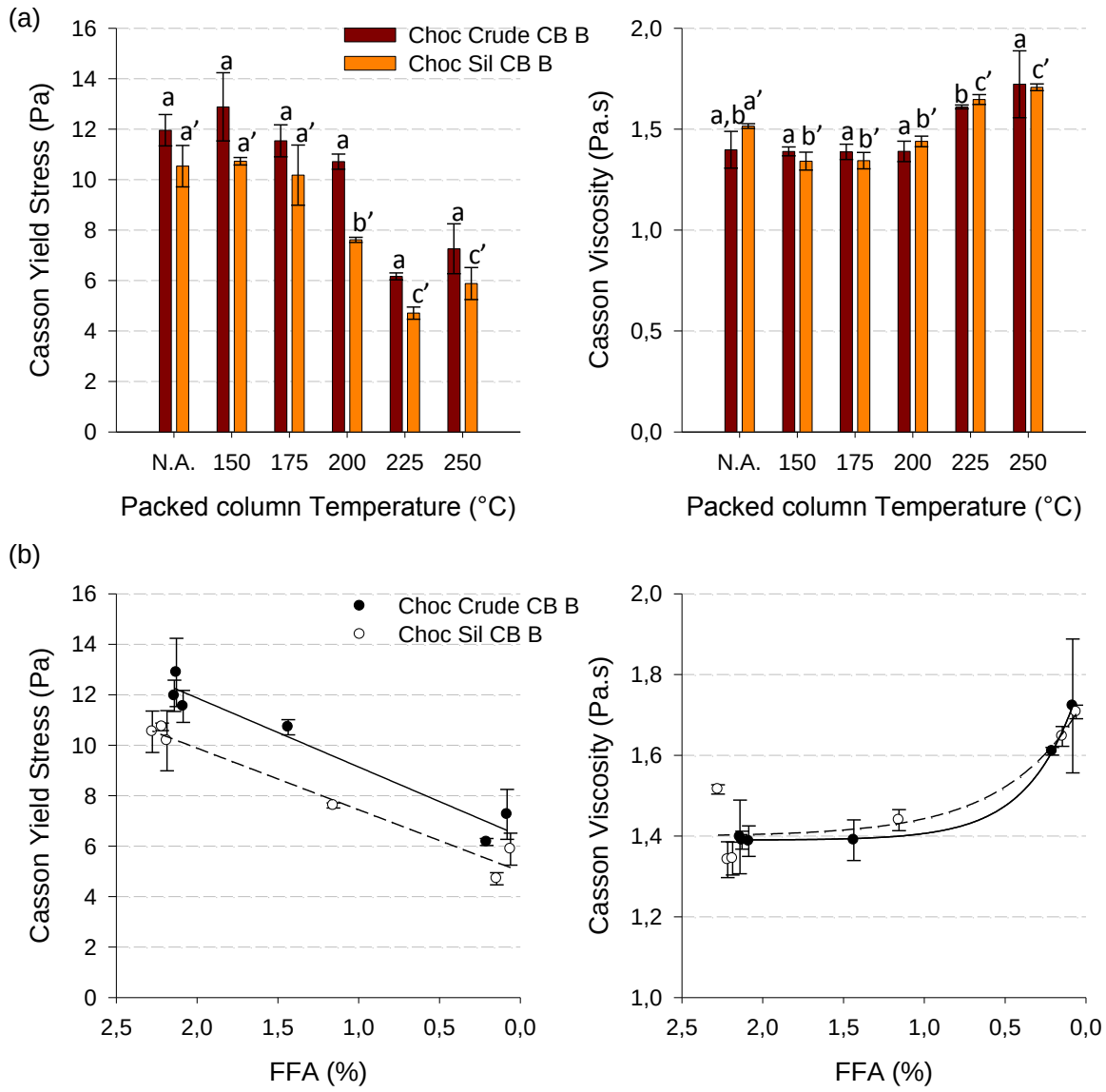


Figure 2.11 Casson yield stress and viscosity as a function of (a) packed column temperature and (b) FFA (%) for chocolates based on crude, silica pretreated and/or packed column steam refined cocoa butter B; linear regression curves for Casson Yield stress R^2 =0.95 (solid line) and R^2=0.97 (dashed line); exponential decay curves for Casson Viscosity R^2=0.99 (solid line) and R^2=0.83 (dashed line); values indicated with same letters are not significantly different (α=0.05); a b: series choc CB B; a' b' c': series choc Sil CB B

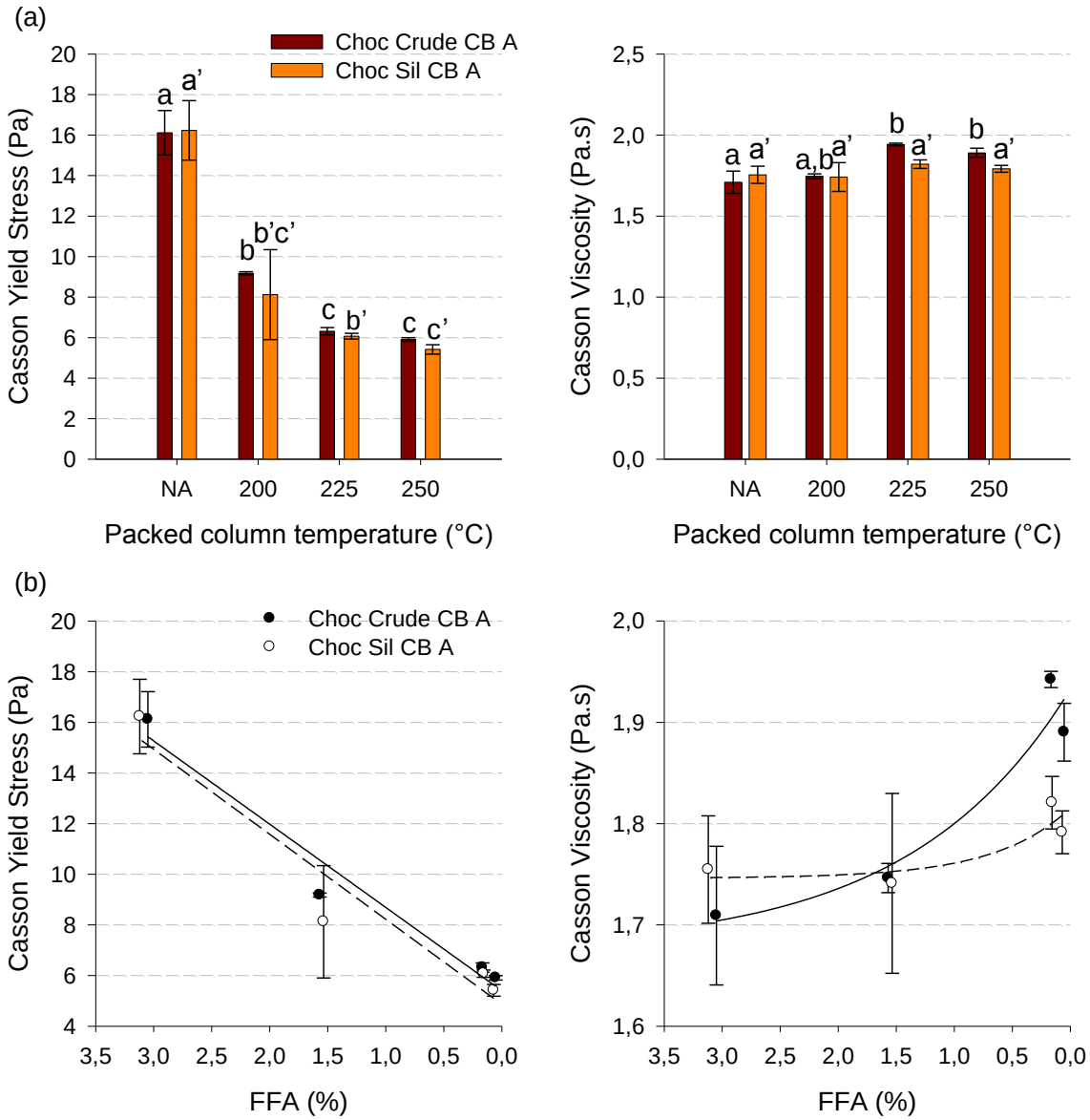


Figure 2.12 Casson yield stress and viscosity as a function of (a) packed column temperature and (b) FFA (%) for chocolates based on crude, silica pretreated and/or packed column steam refined cocoa butter A; linear regression curves for Casson Yield stress R^2 =0,96 (solid line) and R^2=0.93 (dashed line); exponential decay curves for Casson Viscosity R^2=0.93 (solid line) and R^2=0.77 (dashed line); values indicated with same letters are not significantly different (α=0.05); a b: series choc CB A; a' b' c': series choc Sil CB A

Table 2.11 Casson yield stress (σ_{Ca}) and viscosity (η_{CA}) for the chocolates based on crude, silica pretreated and/or packed column steam refined at 250°C cocoa butter C

CB C	σ_{Ca} (Pa)	η_{CA} (Pa.s)
Choc Crude CB	12.04 ± 0.67	1.47 ± 0.03
Choc Sil	11.05 ± 0.62	1.38 ± 0.02
Choc Crude 250°C	5.44 ± 1.49	1.69 ± 0.16
Choc Sil 250°C	5.26 ± 0.09	1.80 ± 0.02

2.4.3.3.3 Discussion

- *Effect of silica pretreatment*

The silica pretreatment removed phospholipids and soaps from the cocoa butter. The phospholipids are amphiphilic molecules that can form a layer between the sugar particles and the fat phase by attaching their hydrophilic part to the sugar particles and the lipophilic to the fat phase. Beckett (2009a) explains that an increase in the amount of lecithin will reduce the viscosity and the yield stress. So the removal of amphiphilic molecules during the silica pretreatment could therefore influence the rheological parameters. The results in Figure 2.11 and 2.12 demonstrate that the Casson viscosity was not significantly different for the samples with or without preceding silica pretreatment at the same packed column temperature. For the Casson yield stress only differences were observed for at 200°C and 225°C for the chocolates based on CB B. At these temperatures, the silica pretreatment resulted in significant lower yield stress values. The amount of lecithin added during chocolate production process (0.4%) most probably was dominant to the naturally present amphiphillic molecules (±0.13% on ±25% cocoa butter in recipe, ±0.03% on chocolate base). So overall, it can be said that the effect of the silica pretreatment on chocolate flow behaviour was limited.

- *Effect of temperature during steam refining*

The temperature applied during steam refining had a more pronounced effect on the flow properties of the produced chocolates. The yield stress decreased at packed column temperatures higher than 175°C; viscosity showed an increasing trend at temperatures ≥ 200°C. As earlier stated, the main effect of the steam refining was the FFA removal. Hence, it was likely to point out these components as the culprit for the observed changes in the flow properties. The Casson yield stress and the Casson viscosity were therefore again plotted as function of the FFA removal. A linear relation was found between the Casson yield stress and the amount of FFA as well as for CB B as for CB A (R^2>0.93).
For the viscosity an exponential increase as function of %FFA removal was observed. This function fitted very well for the chocolates produced with the non silica pretreated butters (R^2>0.9), lower R^2 (R^2=0.83 and R^2=0.77 for CB B and CB B respectively) were found for their counterparts with silica pretreatment. These observations will be discussed in more detail in the following paragraphs.

The present FFA have a quite long C-chain with a polar ester group at the end. Thus they can be considered as amphiphilic molecules. Nevertheless, their emulsifying capacity will be

much lower compared to the commonly used emulsifiers lecithin or polyglycerol polyricinoleate (PGPR).

An emulsifier adsorbs at hydrophilic solid particles (predominantly sugar, milk and to a smaller extent the cocoa particles) into a monomolecular layer, promoting the dispersion of the solid particles into the continuous fat phase and preventing their aggregation. The smaller the solid particles aggregate, the smaller the volume fraction and the lower the plastic viscosity. When solid particles are coated with lecithin molecules (up to a 0.5% lecithin), these latter prevent frictions between them and the yield stress diminishes. These observations by Ghorbell et al. (2011), illustrated in Figure 2.13, confirmed the earlier findings of Schantz and Rohm (2005) and Chevally (1991).

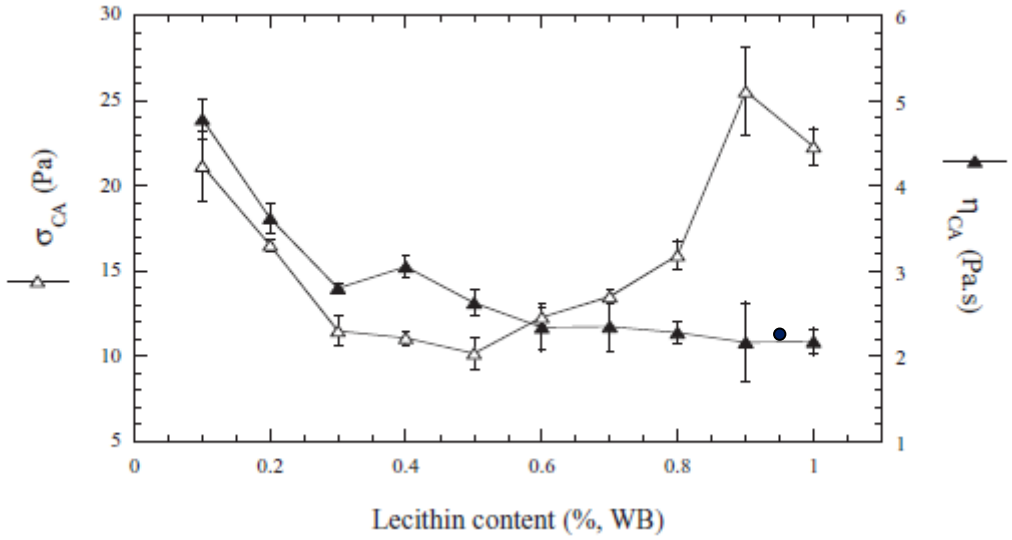


Figure 2.13 Yield stress and plastic viscosity as function of lecithin concentration in a chocolate with a total fat content of 32% (Ghorbel, 2011)

Beyond 0.5 % a new network is created by the excess of phospholipids (Ghorbel et al., 2011). Lecithin particles attach themselves to each other forming new particles, called micelles. Lipophilic ends may attach themselves to lipophilic ends of a second layer of lecithin, bilayer formation, reducing its effectiveness (Beckett, 2009a).

It is difficult to predict how the amphiphilic free fatty acids behave in the chocolate suspension. Therefore, only some hypotheses can be formulated. Similar to the mechanism described above, the free fatty acids can “connect” to the lecithin molecules, forming a type of network. Another possibility is that the FFA act as an emulsifier that interacts with the present particles (cocoa, sugar, milk). In both cases, the FFA will negatively influence the

efficiency of the lecithin. Removing of the FFA during steam refining, removes this hindrance and so yield stress decreases.
Another hypothesis is that the FFA react with other components, like alkaline components to form FA adducts. The reaction products could have surface-active properties negatively influencing the yield stress.

Beyond the yield stress a transition from elastic to viscous deformation is observed. The interactions between the aggregates disrupt progressively, enhancing the flow and decreasing the viscosity. From Figure 2.13 it was expected that, if the FFA are considered as emulsifiers, that the influence on viscosity would be limited. However, all the chocolates showed a significantly higher Casson viscosity when the cocoa butter was refined at packed column temperatures of 200°C or higher. This is again related to the removal of the FFA.
The removal of the FFA is also corresponding with an increase of the amount of TAG in the CB. In the crude, silica pretreated or mildly refined samples, more FFA were present (e.g. ±2 % on cocoa butter base, ±0.5 % on chocolate base) resulting in less CB less TAG. So removing FFA (refining at the higher temperature), increased the amount of free fat on product base with ±0.5%. Notwithstanding that the effect of additional fat is much greater at lower total fat contents (<30%) (Beckett, 2009a) and the chocolate produced in the setup had a total fat content of 33%, the observed increase and more specifically the role of the FFA remained unclear.

The viscosity of the chocolate is mainly determined by the continuous fat phase. Therefore it seemed useful to determine the flow behaviour of the refined butters. The flow behaviour of the crude and silica pretreated samples and their steam refined counterparts at 250°C was determined. The crude and silica pretreated samples from CB B and their steam refined counterparts at 250°C were selected. Their Casson viscosity was measured but no significant differences were observed (results not shown). This pointed out that the effect on the viscosity was an interface related phenomena.
To conclude, when FFA are present a higher stress is necessary before the chocolate starts to flow but once it flows a lower viscosity will be observed.

2.4.3.4 Colour

The colour of the prepared chocolates was measured and the values varied between following borders for chocolates based on CB B: 28.78 – 29.81 for L*; 87.84 – 89.88 for a*; 9.01 – 9.99 for b*. The differences were very small hence the colour differences can't be observed by the human eye. Therefore, in contrast with the colour variation in the refined cocoa butters of CB B (section 2.4.2.4), no distinct colour differences were observed between the corresponding chocolates. Thus, the brown colour of the cocoa powder became predominant. The colour difference between the refined cocoa butters probably will only be distinct when applied to white chocolate production.

2.4.3.5 Texture analysis

The maximum load to fracture a bar of chocolate or the fracturability was measured by a three point bend test. The fracturability is expressed by Szczesniak (2002) as the force with which a material fractures. It is the result of a high degree of hardness and a low degree of cohesiveness. So this parameter combines the force required to compress a substance between molar teeth and the degree to which a substance is compressed between the teeth before it breaks.

Afoakwa et al. (2008b) concluded that attaining an optimal temper regime during tempering of dark chocolate is necessary to achieve high quality products. The degree of tempering affects mechanical properties, appearance, microstructure and melting characteristics. For that reason the influence of different temper regimes on the fracturability of milk chocolate was studied first. Tempering is a shear-temperature-time process, a pre-crystallization at which a small proportion of TAG will form nuclei (1 – 3%) (Afoakwa, 2010; Windhab, 2009). The degree of pre-crystallization was analyzed with a temper meter. It records a cooling curve from which the course of the curve defines the appropriateness of the tempering like described in section 1.7.6. A temper index (TI) of five corresponds to slope zero (cooling is equal to heat release during crystallization) and this is characteristic for a well tempered chocolate. The TI gives an idea of the amount of crystals formed, but not on the type of polymorph.

The reference chocolate (containing CB ref.) was hand tempered in such a way that TI between four and six were obtained. The CTU varied between 22 – 23°C. The three point bend tests on the chocolate bars were performed after one week storage at 20°C. The results are presented in Figure 2.14.

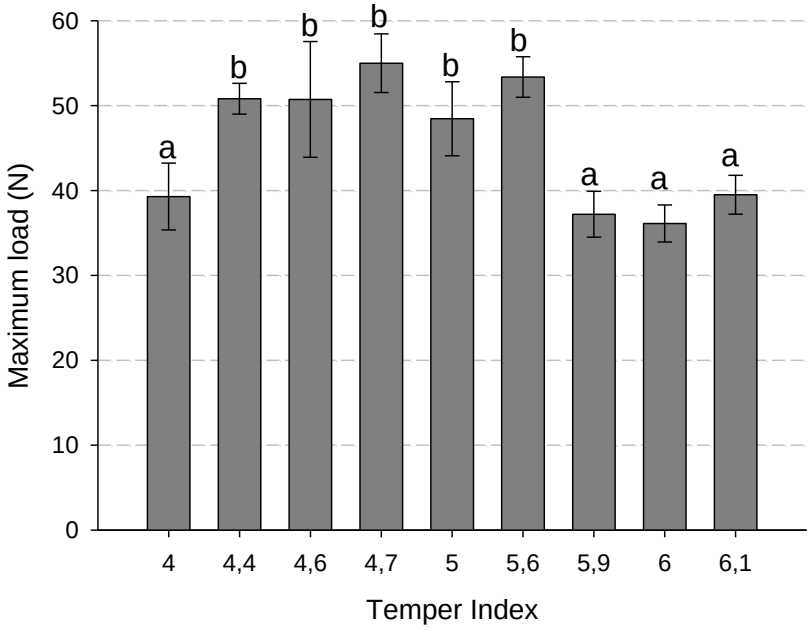


Figure 2.14 Maximum load as function of the tempering index, samples that are not significantly different are marked with the same letter (α=0.5)

It was observed that the maximum load to fracture the chocolates with a TI between 4.4 and 5.6 were not significantly different, values varied around 50 N. Beyond these boundaries, the maximum load to break a chocolate bar was significantly lower. These findings were opposite to the observations of Afoakwa et al. (2008b) who observed the lowest hardness for the well-tempered chocolate. Although one should keep in mind that conclusions were drawn for a dark chocolate and another type of texture analysis was used. According to the same authors, undertempering results in a dissolution of small crystals, rearrangement and re-crystallization into a small number of larger fat crystals (Ostwald ripening) with weak and fewer intercrystal connections within the chocolate structure. For a well tempered chocolate, an even spatial distribution of numerous small stable β-polymorph crystals in a network with well defined interparticle connection was observed (Afoakwa et al., 2008b). So, it can be expected that the network formation in the undertempered chocolates is weaker, less efficient compared to the well tempered chocolate, resulting in a lower maximum load to break the chocolate like for example TI lower or equal to 4 in this experimental setup (Figure 2.14). For the overtempered chocolate, Afoakwa et al. (2008b) observed growth of seed crystals from the melt into submicron primary crystallites and a fat network stabilized by van der Waals forces resulting from a large number of stable β-polymorphs with early nucleation. In an overtempered chocolate, many seed crystals are formed, so crystallization will occur faster. This leads to the spatial distribution of a dense mass of smaller crystals within a network structure containing mixtures of both well- and ill-defined crystal-to-crystal

connections. (Afoakwa et al., 2009). This implies that an overtempered chocolate has a lower fracturability.

The maximum load to break the chocolates with a TI between 4.4 and 5.6 was not significantly different. Therefore, the chocolates in this study were uniformly tempered and it was aimed to obtain a TI between these boundaries. However, it should be recognized that chocolates with a different cocoa butter composition could show different sensitivity towards the TI.

The fracturability of the chocolates produced with the refined CB B samples are presented in Figure 2.15. The analysis was repeated 10 times due to the high variability on the measurements. The relative standard deviation varied around 10%, therefore it was difficult to detect significant differences. The test was performed after a storage period of one and two weeks after production. The measurements after two weeks storage were not significantly different compared with the measurements of week 1.
The silica pretreatment did not result in significant different maximum load values. As function of packed column temperature, the maximum load increased. This was the most evident for the CB with silica pretreatment although the maximum load dropped again at the highest packed column temperature. The comparison of chocolates made with the crude or silica pretreated vs. packed column 250°C for CB A and C confirmed this trend. Possibly, this was again related to the FFA removal. It was concluded in section 2.4.2.6 that crystallization kinetics were affected by this removal. At lower FFA concentrations, cocoa butter tends to crystallize sooner, faster and more solid material will be formed. So the presence of FFA will influence the chocolate microstructure with observed differences on a macroscopic scale. A lower amount of FFA resulted in a stronger crystal network, resulting in a higher maximum load to break and compress a chocolate bar.

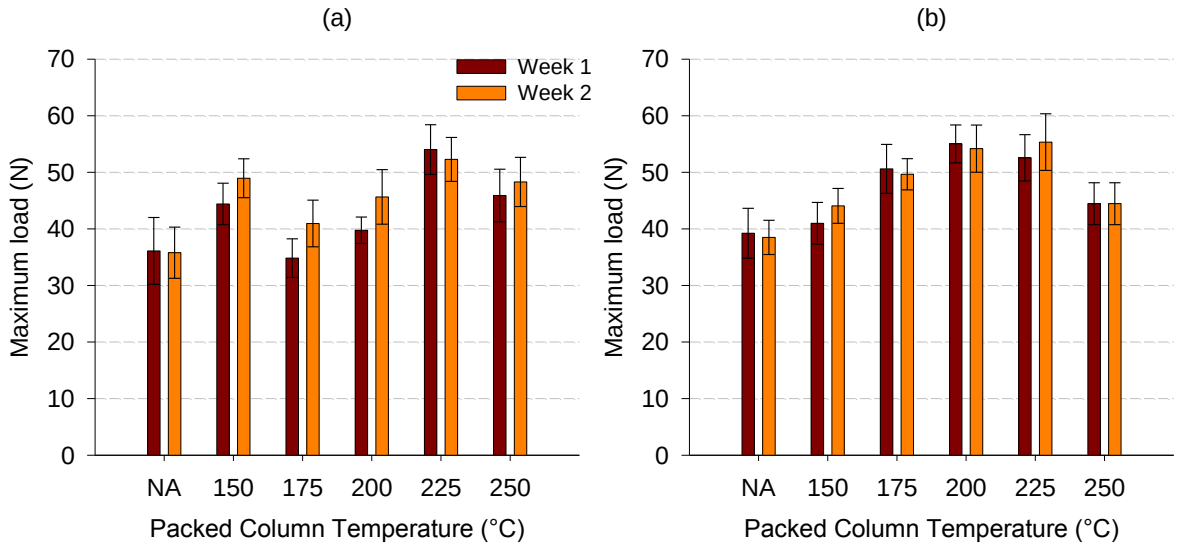


Figure 2.15 Fracturability as a function of packed column temperature measured 1 week and 2 weeks after tempering for (a) Crude CB B; (b) Sil CB B

2.5 Conclusions

The silica treatment and/or packed column steam refining significantly changed the quality characteristics of the treated cocoa butter. The major effect of the silica pretreatment was the complete removal of phosphorous (thus phospholipids), iron and alkaline components. The removal of the pro-oxidant iron almost doubled the oxidative stability. Also a bleaching effect was obtained, resulting in a more yellow butter. Compared to a traditional bleaching process, not all the alkaloids, like theobromine and caffeine, were eliminated. The removal of the alkaloids during this steam refining can cause problems as these deposit on the vapour scrubbers, decreasing its efficiency

The main effect of the steam-refining step was the removal of the FFA at increased temperatures (T≥ 200°C). The changes in physical properties were mainly related to the removal of these FFA. They retarded and slowed down the crystallization and besides the effect on crystallization kinetics a lower solid fat content was obtained at equilibrium conditions.

The impact of the refining conditions on milk chocolate quality were verified. The refining of cocoa butter largely influenced the rheological properties of the chocolate. This is an important finding as chocolate flow properties are very important in chocolate manufacturing. Adjusting the flow properties of molten milk chocolate allows the chocolate manufacturers to optimize their processes and formulations and to reduce the costs. It was observed that an increased packed column temperature, coinciding with the removal of FFA, resulted in a lower yield stress and a higher viscosity. The FFA played a predominant role, probably at the

interface between particles and the continuous phase, but the exact mechanism remains unclear. In the future, a more fundamental study could help to elucidate the observed effects. The FFA influenced the crystallization kinetics and so the formation of the crystal network, resulting in differences on a macroscopic scale.

It can be concluded that by tuning the refining conditions (pretreatment or no pretreatment, temperature of the refining process), the cocoa butter properties can be manipulated to suit the desired chocolate properties.

Chapter 3

Production and purification of cocoa butter diacylglycerols

If any man has drunk a little too deeply from the cup of physical pleasure; if he has spent too much time at his desk that should have been spent asleep; if his fine spirits have become temporarily dulled; if he finds the air too damp, the minutes too slow, and the atmosphere too heavy to withstand; if he is obsessed by a fixed idea which bars him from any freedom of thought: if he is any of these poor creatures, we say, let him be given a good pint of amber-flavored chocolate… and marvels will be performed.

Anthelme Brillat-Savarin (1755 – 1826)

3.1 Introduction

Diacyglycerols (DAG) are natural components of various edible oils and are mostly used in foods as an emulsifier (Matsuo, 2004). In early 1999, Kao Corporation introduced a novel application of DAG oil on the Japanese market because they found that DAG oil had some nutritional advantages compared to TAG. Since then, there has been increased interest for these components. Patent literature reports the use of DAG in numerous applications such as cooking oil, frying oil, salad oil, dressings, shortening and margarines, chocolates, ice cream fats, confectioner's fats, specialty oils with enriched fatty acids, fried and baked food products, etc. (Lo et al., 2008).
In the introduction the physicochemical properties of DAG and the nutritional benefits will be discussed, followed by an overview of the methods to produce DAG. Finally, short path distillation is described as a method to separate acylglycerols.

3.1.1 Physicochemical properties

DAG are esters of glycerol in which two of the hydroxyl groups are esterified with fatty acids. They occur in two isoforms, i.e. 1,2 (or 2,3)-diacyl-*sn*-glycerol (1,2-DAG) and 1,3-diacyl-*sn*-glycerol (1,3-DAG) (Yang et al., 2004). DAG consist mainly of the 1,3-species due to the migration of the acyl group in an equilibrium reaction. Finally the ratio of 1,3-DAG to 1,2(2,3)-DAG is around 7:3. The nature of DAG is more hydrophilic and water soluble than TAG but the difference in energy value between a DAG oil (87% DAG) and a TAG oil with the same fatty acid composition is negligible (Tada, 2004). DAG are typical indigenous minor components. In cocoa butter they account for 1.1 – 2.8% while in palm oil they can be considered as a significant component as levels from 6 – 9% are observed (Timms, 2003).

In general, the melting point of the 1,3-DAG is higher than that of the corresponding TAG and the melting point of the 1,2-DAG is approximately 10°C lower than the 1,3-DAG of the same fatty acid composition. The melting point of some monoacid DAG and their corresponding TAG are presented in Table 3.1. The melting point drops as function of number of double bonds (Bockisch, 1998). The causes of these melting point differences are the strength of hydrogen bonding of the hydroxyl group and fatty acid chain arrangement of the DAG isomers. 1,3-DAG has a V-shaped fatty acid arrangement, while the 1,2-DAG has a hairpin conformation (Figure 3.1). The type of molecular arrangement of the DAG isomer relates to its polymorphic form. Unlike TAG polymorphism, monoacid DAG exhibits two types of polymorphic forms. The 1,2-DAG isoform exhibits the α- and β'-forms but has no β-form. The majority of 1,3-DAG are β-tending, crystallizing in either high-meting β_1 or low-melting β_2

forms. There are however exceptions, for example, diacid 1,3-DAG containing both short- and long-chain acyl groups (e.g. 1,3-acetoyl-stearoyl-*rac*-glycerol) are reported to be β'-tending (Craven and Lencki, 2011; Lo et al., 2008; Nakajima et al., 2004). The polymorphism of DAG has been reviewed by Hagemann (1988) and Shannon (1992). Di and Small (1993) investigated the polymorphic behaviour of mixed chain DAG. 1-stearoyl-2-oleoyl-sn-glycerol was shown to have eight polymorphs: γ2, γ1, α, β_4, β_3, β_2, β_1 and β' in order of increasing melting point. In this case the β'-polymorph is thus the most stable. On the other hand, 1-stearoyl-2-linoleoyl-sn-glycerol shows only four polymorphic phases: α, sub-α (1), sub-α (2) and β'. From this large number of polymorphic forms with similar energy, it can be deduced that the two chains in these mixed chain DAG, have difficulty in deciding what is the most favourable configuration (Di and Small, 1995).

Table 3.1 Melting points of single-acid triacylglycerols and diacylglycerols, β-modification (after Craven and Lencki, 2011[a]; Bockisch, 1998[b]; Shannon et al.,1992[c]; Hagemann, 1988[d])

	Melting point (°C)		
Fatty acid (C_n)	C_n- C_n- C_n	C_n- OH- C_n (β_2)	C_n- C_n- OH (β')
$C_{6:0}$	-25[b]	12.95[a]	
$C_{8:0}$	8.3[b]	37.83[a]	
$C_{10:0}$	31.5[b]	44.96[a]	~27[b]
$C_{12:0}$	46.5[b]	53.80[a]	40.8[b]
$C_{14:0}$	57[b]	52.31[a]	55.5[b]
$C_{16:0}$	65.5[b]	69.46[a]	66.4[c]
$C_{18:0}$	72.5[b]	78.2[b]	71.0[d]
$C_{18:1}$	4.9[b]	36.51[a]	
$C_{18:2}$	-13.1[b]	-2.6[b]	
$C_{18:3}$	-24.2[b]	-12.3[b]	

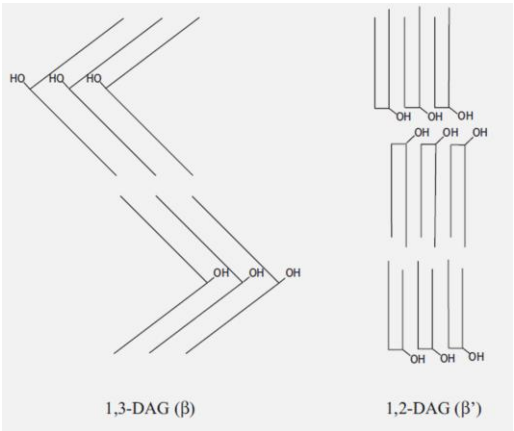


Figure 3.1 Crystal arrangement of β-form 1,3-DAG and β'-from 1,2-DAG (Lo et al., 2008)

3.1.2 Nutritional characteristics and health benefits

3.1.2.1 Digestion, absorption and metabolism

DAG can be digested by the same gastrointestinal enzymes that hydrolyze TAG. However, upon digestion, DAG does not follow the resynthetic pathway of TAG, which include the 2-monoacylglycerol (2-MAG) and the glycerol-3-phosphate (GP) pathway. During digestion, TAG are hydrolyzed by 1,3-lipases to 1,2-DAG and 2,3-DAG, because lipases cleave only at the 1 or 3 position. Supplementary action of lipase on 1,2-DAG and 2,3-DAG leads to 2-monoacylglycerol and free fatty acids. These are the normal end products of TAG digestion that are absorbed by intestinal mucosal cells and are used for the reconstruction of circulating chylomicron TAG (Figure 3.2a). In case of DAG, however, the 1(3)-MAG are poorly reesterified via the 2-MAG pathway and thus have an insignificant contribution towards resynthesis. Instead, substantial amounts of 1(3)-MAG are further hydrolyzed to free fatty acids and glycerols which may be less readily resynthesized to chylomicron TAG. Larger amounts of fatty acids from digested DAG may be released into portal circulation rather than being incorporated into chylomicrons. In addition, this hepatic exposure to fatty acids by increasing DAG intake may lead to greater β-oxidation by the liver than that after TAG intake (Lo et al., 2008; Rudkowska et al., 2005). This potential mechanism is illustrated in Figure 3.2b.

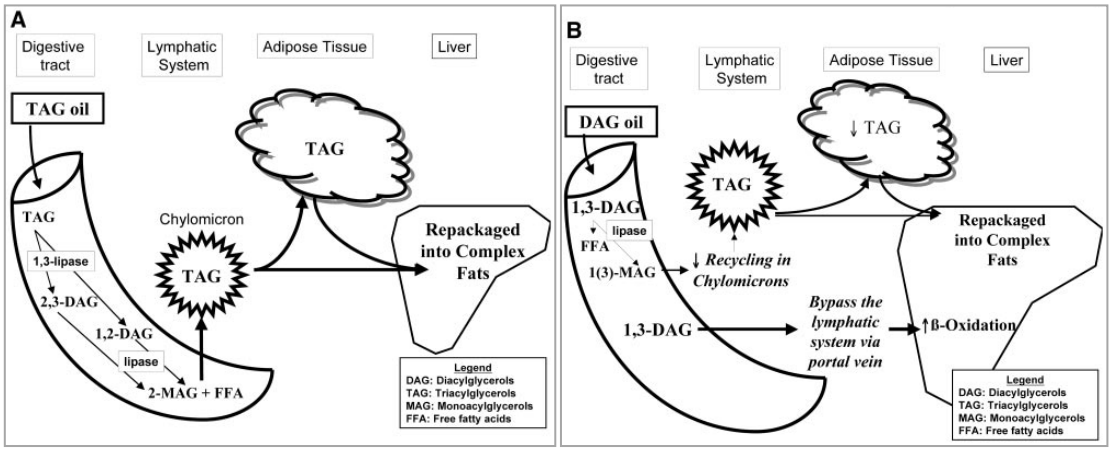


Figure 3.2 (A) Mechanism of action of triacylglycerols and diacylglycerols including 2,3-DAG and 1,2-DAG isomers (B) Mechanism of action of DAG oil including 1,3-DAG isomer (Rudkowska et al., 2005)

3.1.2.2 Potential health benefits

The beneficial effects of DAG compared to TAG are probably caused by its distinct metabolism after the absorption like described in the previous paragraph, but not by its different bioavailability (Matsuo, 2004). Studies in animals and humans indicate that diets

containing DAG oil (containing >80% DAG) decrease body weight gain and body fat accumulation, especially visceral fat (Lo et al., 2008). Body weight and body fat are controlled by energy expenditure, fat oxidation, fat storage capacity, and appetite control. Short-term human studies with indirect calorimetry demonstrated higher fat oxidation with DAG oil consumption compared with TAG oil consumption. Furthermore, DAG oil consumption for 14 days stimulates energy expenditure. Based on these reports, enhanced fat oxidation and energy expenditure by daily DAG oil intake could contribute to long-term reductions in body weight and fat accumulation (Hibi et al., 2009).

Nevertheless, the effects of DAG on body weight are not consistent in clinical trials. Therefore a meta-analysis was performed and this suggested that DAG were efficacious for reducing body weight compared with TAG and that this effect was influenced by the daily dose (Xu et al., 2008). Also, studies investigating the effect of DAG oil on serum TAG levels in animals and humans showed inconsistent results, as illustrated by Lo et al. (2008).

DAG are a better solvent for phytosterols than TAG. It is known that phytosterols reduce plasma total cholesterol and LDL cholesterol. Megoru et al. (2003) demonstrated that phytosterols in combination with DAG, as compared to TAG, prevent the development of atherosclerosis by decreasing the total serum cholesterol in rabbits.

Morita and Soni (2009) concluded that there is sufficient qualitative and quantitative scientific evidence available from animal and human studies suggesting that the intake of DAG oil is safe for human consumption when used in a manner similar to other edible oils.

An extensive review of the animal and human studies on the potential benefits of 1,3-DAG, performed before 2005 can be found in Rodbowska et al. (2005). More recent data describing the nutritional aspects of DAG can found in Lo et al. (2008), Yanai et al. (2008), Xu et al. (2008) and Hibi et al. (2009).

3.1.3 Production of DAG

Various routes for the production of DAG have been reported in literature. In general, DAG can be produced via (Lo et al., 2008):

- Glycerolysis between TAG and glycerol, chemical or enzymatically (G_C or G_E), with or without organic solvent
- Esterification of fatty acids or its derivatives to glycerol (E), with or without organic solvents
- Selective hydrolysis of TAG (H)
- Combination of the above described methods

Table 3.2 gives an overview of the substrates, applied reaction conditions and obtained yields for producing (1,3-)DAG as found in literature. As reaction conditions were often optimized in the different studies, Table 3.2 lists the most optimum conditions to produce the DAG. Besides scientific literature, also a lot of processes to produce DAG rich products have been patented. A good overview of patent literature related to this subject is given by Lo et al. (2008).

The synthesis of partial acylglycerols has been thoroughly studied, mainly aiming at high MAG yields. As can be seen from the Table 3.2, glycerolysis is frequently used as a tool to produce DAG. This reaction involves the removal of an acyl moiety from the TAG molecule and an acylation of MAG formed during the reaction (Cheong et al., 2007). The chemical glycerolysis is executed at high temperatures (>200°C) in the presence of an inorganic catalyst (like NaOH). However, these high temperatures are unattractive from a production point of view especially for the heat sensitive polyunsaturated fatty acids (Zhong et al., 2010). Therefore, the enzymatic alternative is preferred as it's a very simple process. The oil/fat and glycerol are mixed with the enzyme at mild temperatures, without the need of solvents yielding between 40-60% DAG. Selection of the enzyme and particularly their carrier material, is crucial to avoid clumping of enzyme particles during the reaction (Kristensen et al., 2005a). Recently ionic liquids came into the picture to develop an efficient reaction protocol for DAG production (Kahveci et al., 2009).

In lipase-catalyzed esterification, DAG are synthesized through esterification of fatty acids and glycerol with simultaneous removal of water by vacuum and molecular sieves. A 1,3-specific enzyme is often used to produce high-purity 1,3-DAG. The raw material (i.e. the fatty acids) is often first produced by partial hydrolysis or a deodoriser destillate (cheap byproduct from the deodorization process) can be used as a starting source (Table 3.2).

The hydrolysis reaction to produce DAG, should be carefully controlled to avoid complete hydrolysis (Coteron et al., 1998) and is therefore less applied for DAG synthesis.

Table 3.2 Methods for the production of DAG

Type*	Substrate**	Reaction conditions***	Yield	Reference
G_C	Butterfat	200°C, 120 min, NaOH	60% MAG, 40% DAG	Campbell-Timperman et al. (1996)
G_E	Olive oil	80°C, 7 h, 600 rpm, Novozym 435	~30% DAG	Coteron et al. (1996)
G_E	Soybean oil	40°C, 1 h, ratio 1:2, 1% *Pseudomonas*	~40% DAG	Noureddini and Harmeier (1998)
G_E	Fish oil	60°C, 24 h, 300 rpm,ratio 1:1.5, 10 %Chirazyme L2, 10%	~40% DAG	Torres et al. (2002)
G_E	Triolein	55°C, 24 h, 200 rpm, ratio 1:2, hexane/octane, Lipozym IM77	~40% 1,3-DAG	Liao et al. (2003)
G_E	Rapeseed oil	60-65°C, 4-5 h, 250 rpm, rato 2:1, 5% Novozym 435	~60%	Kristensen et al. (2005b)
Gc	Butter fat	200°C, 120 min, 0.1% NaOH	~40%	Yang et al. (2004)
G_E	Palm olein	20-60°C, 22 h, 200 RPM, ratio 1:1.5, 10% Lipopzym RM IM, Novozym 435	~45%	Yeo et al. (2009)
G_E	Soybean oil	70°C, 24 h, 300 RPM, ratio 1:8, 2% *Candida antarctica,*	~47%	Fregolente et al. (2009)
G_E	Triolein	60°C, 48 h, 700 RPM, ratio 2:1, 10% Novozym 435, ionic liquids	~50%	Kahveci et al. (2009)
Gc	Soybean oil	35-55°C, 2-4 h, 800 RPM, 0.4wt% NaOH, tertiary alcohol, acetone	~52%	Zhong et al. (2010)
G_E	Triolein	55-60°C, 24 h, 700 RPM, ratio 2:1, 15% Novozym 435, ionic liquid	~70%	Kahveci et al. (2010)
G_E	Tuna oil	40°C, 24 h, 500 RPM, ratio 1:3, 10% Lipozym RI IM, 10% water	~70%	Eom et al. (2010)
E	FFA, FA alkyl esters, FAVE	20°C, 48 h, 10% Lipozyme, different organic solvents	>80% 1,3-DAG	Berger et al. (1992)
E	FFA	25-45°C, 12 h, ratio 2:1; 4%, Lipozyme RM IM, partial vacuum	max 84 1,3-DAG%	Rosu et al. (1999)
E	FFA	50°C, 450 RPM, ratio 2:1, 5% Lipozyme RM IM, vacuum	84% 1,3-DAG	Watanabe et a. (2003)
E+G	MAG, Rapeseed oil, FFA	60°C, 2 h, 5% Lipozyme RM IM vacuum	60-70% DAG	Weber and Mukherjee (2004)
E	Palm oil deodorizer distil.	65°C, 6 h, 800 RPM, ratio 2.5:1, 30% molecular sieves, 10% Lipozyme RM IM	~52% DAG	Lo et al. (2004a)
E	Corn oil deodorizer distil.	65°C, 5h, 800 RPM, ratio 2.5:1, 30% molecular sieves, 10% Lipozyme RM IM	~70% DAG	Lo et al. (2004b)

Continuation Table 3.2

Type*	Substrate**	Reaction conditions***	Yield	Reference
E	Soybean oil based FFA	50°C, 2 h, ratio 2:1, vacuum packed Lipozyme RM IM	~70% DAG	Watanabe et al. (2005)
E	Conjugate Linoleic acid	45-55°C, 3 h, ratio 2:1, vacuum, N_2 bubbling, 7% Novozym 435	94% 1,3-DAG	Guo and Sun (2007)
E	Mixed FFA	60°C, 6 h, 900 RPM, ratio 2:1, vacuum, 1.8% Novozym 435	~60% DAG	Tangkam et al. (2008)
E	Oleic acid	60°C, 12 h, 200 RPM, ratio 2.5:1, 10% Novozym 435, t-butanol, molecular sieves	40% 1,3- DAG	Duan et al. (2010)
H	Triolein	40°C, 250 RPM, pancreatic lipase, phosphate buffer	79% DAG+MAG	Plou et al. (1996)
H	Trilaurin	25°C, diisopropyl ether, *Penicillium roquefortii*	70% 1,2-DAG	Fureby et al. (1997)
H	Palm olein	65°C, 12 h, 50% water, 10% Lipozyme RM IM	32% DAG	Cheong et al. (2007)
H	Soybean oil	35°C, 8 h, 40% water, phospholipase A_1	40% DAG	Wang et al. (2009a)
H	Palm olein	45°C, 8 h, 44% water, phospholipase A_1	40% DAG	Wang et al. (2009b)

* G: glycerolysis, subscript E enzymatic, C: chemical; E: esterification; H: hydrolysis
** FFA: free fatty acids; FAVE: fatty acids vinyl esters
*** molar ratio: substrate/glycerol

3.1.4 Separation

Purification is an essential process to increase the DAG yield. Short-path distillation (SPD) is a good technique to separate the different acylglycerols. SPD is a continuous separation process working under vacuum conditions in which low evaporating temperature and a short residence time allow the distillation of the thermosensitive products with minimal thermal stress. To use distillation as a method for separation, the compounds must be sufficiently volatile before they are decomposed or polymerized at high temperatures. The separation possibility is largely dependent on the difference in vapor pressures between two components at certain temperatures. A typical pressure range is the “fine vacuum range”, i.e., between 1 and 0.001 mbar or even lower. At this pressure, the required evaporation temperature will decrease so that during the short residence time, no thermal decomposition of the product can occur. In SPD, the condenser is located inside a cylindrical evaporator. The distance for the vapors between evaporator and condenser is extremely short, so there is no pressure drop. If the distance between evaporator and condenser is in the order of the average free path length of the molecules, the probability of a vapour molecule colliding with another molecule is smaller than the probability of travelling without collision to the cold

surface. Therefore, this process is also called molecular distillation. In most current publications, the two terms often refer to the same (Xu, 2005).
The feed is admitted into the evaporator under vacuum and is immediately spread into a very thin film. Basically, there are two kinds of molecular distillators: falling film and centrifugal distillators. The first uses gravity force in combination with a wiping element to promote thin film formation while centrifugal distillators use centrifugal force to form this thin film. Heated walls and the vacuum enable the molecules to evaporate from the evaporator to the condenser (distillate) and the less volatile components (residue) continue down the cylinder (Fregolente et al., 2007; Xu, 2005). Operation temperature and residence time are reduced by using SPD which will extend the separation possibility of bioactive compounds.

MAG, DAG and TAG with similar fatty acid compositions have a different boiling point as they contain different numbers of fatty acids in the molecule. Generally, a one or a two-step SPD is included in the purification process. Firstly, fatty acids sometimes together with the MAG, are removed usually at a lower temperature after which the DAG are molecularly distilled from the TAG. Although SPD was applied to separate DAG in some studies (Cheong et al., 2007; Kristensen et al., 2005b; Weber and Mukherjee, 2004; Yang et al., 2004), often no detailed information on minor components, acyl migration and suitable parameters of the process are reported (Wang et al., 2010).

3.2 Research strategy

Cocoa butter is known for its typical physical properties, mainly determined by its relatively simple TAG composition. The most remarkable characteristic is the narrow melting range between 32 – 35°C. In this part of the research the aim was to redistribute these fatty acids to synthesize cocoa butter DAG.
DAG oils have been produced experimentally from different sources of fat and oils e.g. milk fat, soybean oil, palm olein, lard, deodorizer distillates but up to the best of our knowledge cocoa butter has never been used as a substrate. As DAG have distinct physical and nutritional properties compared to the TAG, it was interesting to explore the possibilities of cocoa butter based DAG. Therefore, in a first step it was necessary to define an appropriate synthesis pathway. In literature, several methods are described (Table 3.2). However, the aim was to avoid the use of solvents and to keep the reaction as simple as possible, therefore enzymatic glycerolysis was the method of choice.
Response surface methodology (RSM) allows a reduced number of experiments to be run, but still providing sufficient information for statistically acceptable results (Bas and Boyaci,

2007). For that reason, it was used as a tool to investigate the influence of several variables on the DAG yield.

While the MAG and FFA can be removed by more conventional means, such as refining processes, the separation of high purity DAG from TAG is more difficult. Short-path distillation allows separation of the different acylglycerols based on their different boiling points under vacuum conditions. SPD has been used for a long time in industry for the purification of MAG (for the use as emulsifiers). However, very few publications are available on the separation of DAG from TAG by using SPD. Most of the detailed information on the industrial technology remains unknown to the public (Wang et al., 2010; Xu, 2005). It was therefore necessary to determine the distillation parameters that yield a highly purified CB DAG fraction.

3.3 Materials and methods

3.3.1 Enzymatic reaction

All lab-scale reactions were performed with 50 g of cocoa butter (Barry Callebaut, Wieze, Belgium) in 250 ml flasks with glass stoppers and magnetic stirring (200 rpm) in a waterbath at the desired temperature. The lipase used in all the reactions was Novozym 435 (*Candida antharctica* lipase B immobilized on a macroporous acrylic resin) supplied by Novozymes A/S (Bagsværd, Denmark). Glycerol (99%) was obtained from Across Organics (Geel, Belgium).

For the scaling up of the reaction, a double walled vessel of 500 ml and 5 l, with internal glass sinter filter and mechanical stirring was used. Reactions were followed by vacuum filtration to separate the enzyme from the reaction mixture.

3.3.2 Response surface methodology

Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes in which a response of interest is influenced by several variables and the objective is to optimize this response. RSM has important application in the design, development and formulation of new products, as well as in the improvement of existing product design. It defines the effect of the independent variables, alone or in combination, on the processes. In addition, to analyze the effects of the independent variables, this experimental methodology generates a mathematical model which describes the chemical or biochemical processes (Bas and Boyaci, 2007).

Response surface methodology is a set of techniques that encompasses:

1. Setting up a series of experiments (designing a set of experiments) that will produce adequate and reliable measurements of the response of interest;
2. Determining a mathematical model that best fits the data collected from the chosen design by conducting appropriate tests of hypotheses concerning the model's parameters;
3. Determining the optimal settings of the experimental factors that produce the maximum (or minimum) value of the response.

If discovering the best value of the response is beyond the goal of the experiment, then response surface methods are aimed at obtaining at least a better understanding of the overall system.

The factors or independent variables are processing conditions or input variables whose values or settings can be controlled by the experimenter. Presumably, if one changes the settings of the factors, the values of the response variables change as well (Calliauw, 2008).

In this design five factors were optimized. A fractional central composite design (CCD) was used as the response surface method. CCD's are designed to estimate the coefficients of a quadratic model. It has three groups of design points (StatEase, 2010):

- Two-level factorial or fractional factorial design points: consists of all possible combinations of the +1 and -1 levels of the factors
- Axial points or also called "star" points: these star points have all of the factors set to 0, the midpoint, except one factor, which has the value +/- alpha
- Center points: points with all levels set to coded level 0

It was decided to vary the factors over three levels. This can be created by setting alpha distance to one. This is commonly referred to as a face-centered central composite design. The design was set up with the software Design-Expert version 5.0 (Stat-Ease, Minneapolis, USA) and generated 36 experiments.

3.3.3 Short-path distillation

The distillation was performed using laboratory short-path distillation VKL 70 unit from VTA GmbH (Germany). The major part of the equipment was constructed from glass and the installation consisted of a short-path evaporator with integral condenser, a cooling trap for liquid nitrogen and a vacuum pump system attaining values up to 0.5×10^{-3} mbar. The evaporator surface was 0.04 m². The enzymatically modified product was preheated at 80°C. The temperature of the internal condenser was also set at 80°C. The evaporator temperature and vacuum pressure were varied to investigate the distillation performance.

3.3.4 Free fatty acids

The FFA contents were analyze by using titration according to the AOCS Official Method Ca 5a-40 (Firestone, 1997) and is expressed as % oleic acid.

3.3.5 Fatty acid methyl ester profile

This AOCS official method Ce 2-66 (Firestone, 1997) was used for preparing methyl esters for further analysis by GC according to the AOCS Official Methods Ce 1-62 (Firestone, 1997).

3.3.6 Acylglycerol analysis by GC

Acylglycerols were separated according to their number of C-atoms by Capillary gas chromatography as described by an internal method of Barry Callebaut.

3.3.7 Acylglycerol analysis by HPLC

The fat sample is dissolved in acetonitrile/dichloromethane 70/30 (v/v). Separation of TAG species was performed on a Thermo Finnigan Surveyor HPLC system with four solvent lines, degasser, autosampler and ChromQuest software (Thermo Electron 2003, Brussels, Belgium) coupled to an Alltech ELSD 2000 evaporative laser light scattering detector (Grace Alltech, Lokeren, Belgium) was used to analyze the acylglycerol profile. A gradient of acetonitrile (VWR, Leuven, Belgium) and dichloromethane (Acros Organics, Geel, Belgium) at 0.72 ml/min was used as mobile phase. N_2 was used as the nebulizing gas at a flow of 1.5 ml/min, and a nebulizing temperature of 45 ºC. The column was a 15 x 93.0 mm Alltima HP C18 HL with 3 µm particle diameter (Grace Alltech, Lokeren, Belgium). A pre-column with a silica packing was used. The flow was 1.5 ml/min, resulting in a back pressure of 95 – 100 bar. 25 µl of a 3.5 mg/ml solution was injected. More information about this in-house developed method can be found in Rombaut et al. (2009). Peak areas were correlated with the concentrations of the different acylglycerols in the sample.

The solvent gradient developed for the analysis of TAG was further optimized for a more optimal separation of the DAG species. The mobile phase gradient was adjusted as described in Table 3.3. This adjustment made it possible to separate the DAG isomers.

Table 3.3 Mobile phase gradient method separation DAG

Time (min)	0	7	17	30	31	32	33	40
Acetontrile	70	64	60	35	30	30	70	70
Dichloromethane	30	36	40	65	70	70	30	30

3.4 Results and discussion

3.4.1 Development of the enzymatic glycerolysis reaction

The goal of this research was to enzymatically modify CB, to produce DAG for the application in chocolate products.

3.4.1.1 Reaction parameters and enzyme selection

Based on the literature search (Table 3.2), it was decided to use an enzymatic glycerolysis reaction to synthesize the DAG. In this type of reaction the oil or fat can be used directly together with glycerol and the enzyme, without the need to first liberate the FFA. DAG can be formed both by the removal of an acyl moiety from the TAG and by acylation of MAG formed during the reaction. No solvents were used as the intent to used the DAG product in foods makes the use of solvents undesirable (Kristensen et al., 2005a).

To determine the optimal reaction conditions, response surface methodology is often used since it enables observations of interaction effects of multiple parameters on the response. It also offers a large amount of information from a small number of experiments (Cheong et al., 2007). Determination of the independent variables and their levels is an essential step for a successful optimization (Bas and Boyaci, 2007). So, it was necessary to identify the parameters that have major effects. Based on literature (Ferreira-Dias et al., 2001; Kristensen et al., 2005b; Noureddini and Harmeier, 1998) following variables and set boundaries were identified:

- Reaction time: 3 to 15 hours
- Reaction temperature: 40°C – 75°C
- Enzyme load: 3 to 15 wt% of oil mass
- Substrate molar ratio, oil/glycerol: 0.25 – 2.00
- Water content: 0 to 6 wt% of the glycerol mass

The choice of the appropriate enzyme is of utmost importance since it catalyzes the reaction. The studies from Kristensen et al. (2005b) and Ferreira-Dias et al. (2001) both concluded

that the non-specific *Candida antarctica* (commercial name Novozym 435) gave the best result. It's a thermostable enzyme immobilized on a macroporous acrylic resin with a maximum activity in the range 70 – 80°C. The carrier material is important as a hydrophilic carrier can attract glycerol, forming a layer around the enzyme, restricting the contact with the hydrophobic oil phase and thus decreasing the enzyme activity.

3.4.1.2 Response surface methodology

3.4.1.2.1 Experimental results

A fractional Face-Centered Central Composite Design was set up and generated 36 experiments. The factor levels for the five reaction parameters and the corresponding response is given in Annex II. The response, the % DAG, was expressed based on the total amount of acylglycerols.

3.4.1.2.2 Regression calculations

In the regression calculations the software fits all of the polynomial models (linear, quadratic, cubic) to the selected response. The cubic model however has aliased terms, meaning the design matrix provides too few unique point to determine all of the terms (Calliauw et al., 2008). Therefore, this model cannot be taken into further consideration. The best model is then selected based on three statistical criteria: sequential model sum of squares, lack of fit test and model R^2. Based on the results a quadratic model was selected.

3.4.1.2.3 ANOVA results of the quadratic fit

After model fitting, a regression analysis was performed to remove factors and/or interaction which didn't contribute significantly to the model. Parameters can be selected by backward elimination, forward selection and step-wise regression. Forward selection begins with a simple regression model using the single term having the highest correlation with the response and then values are added until the p-value of inserted term in does not meet the specified "Alpha In" value criterion. The disadvantage is that some terms may never get the chance to be included in the model, the same is true for stepwise regression as it also starts with a core model. The backward regression procedure differs from stepwise, but the results are often very similar, if not the same. All blocks and forced terms are fit to the data. Then the remaining candidate factors are considered. The backward method is considered the most robust choice for algorithmic model reduction because all model terms will be given a chance to be included in the model (StatEase, 2010). Based on this, a quadratic model with

backward elimination was used to describe the response as function of five factors. The most important ANOVA results are presented in Table 3.4. The extended output can be found in Annex II-B. The model F-value of 15.03 implies that the model was significant. Values of "Prob> F" less than 0.05 indicated that model terms had a significant effect on the response. In this case A, B, C, D, A*D, B*C, A^2 and D^2 were significant model terms. It can be seen that the first order contribution of factor E, the water content, was removed. This parameter didn't seem to influence the response. The model didn't show a significant lack of fit, although the p-value= 0.08 was quite low. This may indicate that the model was not fully reliable to predict the response (% DAG). The quadratic model showed an R^2 of 0.844, which was acceptable. The Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The value of 16 indicated an adequate signal, so the model can be used to navigate the design space. The predicted results are quite close to the observed. However, deviations were observed for the higher amounts of DAG.

Table 3.4 ANOVA results for quadratic model

ANOVA for Response Surface Reduced Quadratic Model
Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Block	244.79	1	244.79			
Model	1915.93	9	212.88	15.03	< 0.0001	significant
A-Reaction time	*149.01*	*1*	*149.01*	*10.52*	*0.0033*	
B-Enzyme Load	*455.92*	*1*	*455.92*	*32.19*	*< 0.0001*	
C-Temperature	*424.38*	*1*	*424.38*	*29.96*	*< 0.0001*	
D-Substrate molar ratio	*64.00*	*1*	*64.00*	*4.52*	*0.0436*	
AC	*5 0.98*	*1*	*50.98*	*3.60*	*0.0694*	
AD	*141.61*	*1*	*141.61*	*10.00*	*0.0041*	
BC	*119.46*	*1*	*119.46*	*8.43*	*0.0076*	
A^2	*66.20*	*1*	*66.20*	*4.67*	*0.0404*	
D^2	*79.95*	*1*	*79.95*	*5.64*	*0.0255*	
Residual 354.13	25	14.17				
Lack of Fit *301.96*	*17*	*17.76*	*2.72*	*0.0761*	*not significant*	
Pure Error *52.17*	*8*	*6.52*				
Cor Total 2514.86	35					

Std. Dev.	3.76	R-Squared	0.8440
Mean	40.78	Adj R-Squared	0.7878
C.V. %	9.23	Pred R-Squared	0.6852
PRESS	714.70	Adeq Precision	16.126

A high amount of MAG in the reaction mixture can be advantageous in DAG production if TAG are simultaneously decreased, since MAG and DAG are more easily separated than DAG and TAG. MAG can be further used as food emulsifier or be re-used for DAG synthesis (Kristensen et al., 2005b). For this reason, it was tried to use the amount of MAG and the sum of MAG+DAG as a response. Unfortunately, there was a significant lack of fit and the R^2 was considerately low making it impossible define a reliable model.

3.4.1.2.4 Main effects and interactions between the parameters

There are many contributing factors that affect the DAG formation. Table 3.5 shows that the response was positively affected by all four parameters. Among all, the enzyme load had the highest effect followed by temperature, reaction time and substrate molar ratio. The factor "water content" was removed from the model. This means that it was not necessary to add extra water to the reaction mixture to ensure lipase activity. This is in correspondence with the findings of Kristensen et al. (2005b). These authors stated that the macroporous acrylic carrier used for Novozym 435 contained 1 – 2 wt% water, which seemed to be sufficient to achieve catalytic active lipases.

Table 3.5 Regression coefficients and p-values after backward elimination

Variable	Regression coefficients	p-values
Intercept	45.54	0.001
A: Reaction time	2.88	0.0033
B: Enzyme load	5.03	<0.0001
C: Temperature	4.86	<0.0001
D: Substrate molar ratio	1.89	0.0436
AC	-1.79	0.0694
AD	2.97	0.0041
BC	-2.73	0.0076
A^2	-4.39	0.0404
D^2	-4.83	0.0255

The major influence of each parameter on DAG yield was visualized in the perturbation plot or a main effect plot. A perturbation plot helps to compare the effect of all the factors at a particular point in the design space. The response was plotted by changing only one factor over its range while holding all the other factors constant. The reference point was set at the midpoint (coded 0) for all the factors (Figure 3.3). It was deduced that the effect of the enzyme load (B) and the temperature (C) were very similar. They both had a positive linear relation with the response. The maximum yield was situated around 50%. The optimal temperature range for maximum enzyme activity is 70 – 80°C nevertheless a temperature range between 40 – 60°C is recommended by the producer of Novozym 435 to extend its operational stability (Ferreira-Dias et al., 2001). Related to the enzyme load, it has been reported that an increase in the amount of lipase above a certain amount (± 10 wt%) did not increase the yield but might increase the reaction rate. A higher amount of enzyme may lead to poorer mixing and thereby the occurrence of mass transfer limitations, resulting in a lower DAG yield (Kristensen et al., 2005b; Noureddini and Harmeier, 1998). However, this was not

the case in this experimental set-up as a reaction with 15% enzyme (highest level), significantly improved the DAG yield compared to the center level.

Reaction time (A) and substrate molar ratio (D) on the other hand, showed a maximum at their midpoint conditions, 9 hours and 1.13 respectively. This maximum was not observed by Kristensen et al. (2005b). In their study with soybean oil as a substrate, they reported a positive effect of increasing substrate molar ratio on the DAG yield, with the highest yield at the lowest glycerol concentrations.

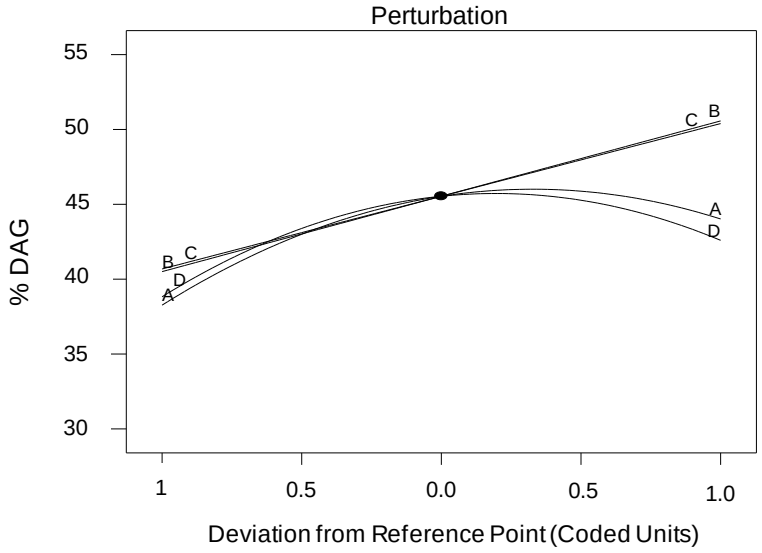


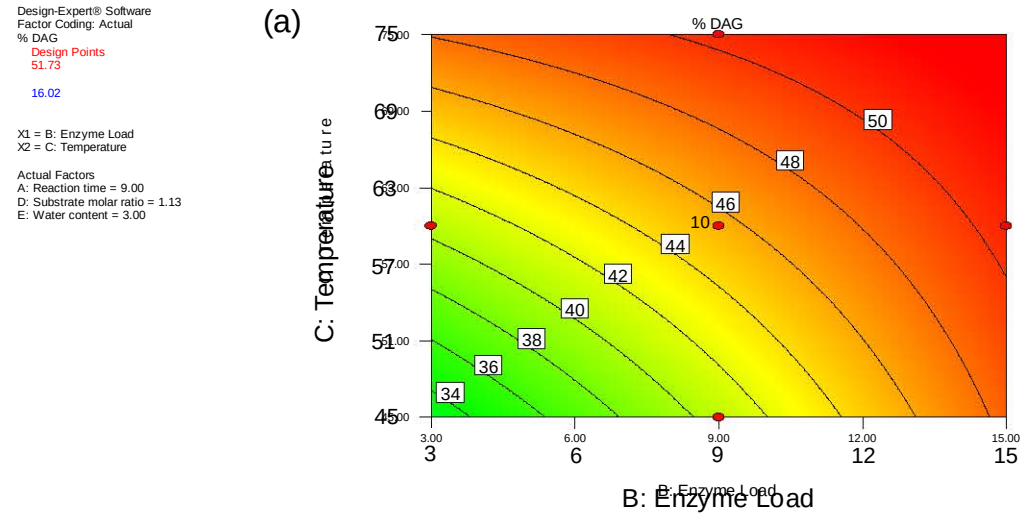
Figure 3.3 Perturbation plot of A: reaction time (9 hours); B: enzyme load (9%); temperature (60 °C); D: substrate ratio (1.13); midpoint value between brackets

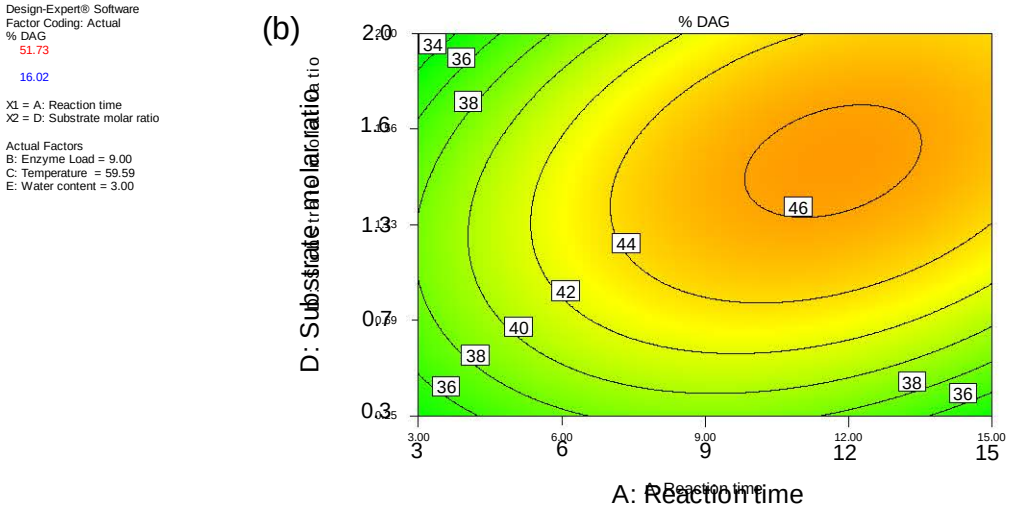
The quadratic terms had a negative effect on the DAG yield, except the interaction between reaction time and substrate molar ratio which showed a positive effect (Table 3.4). The negative interaction between reaction time and temperature was not significant ($p>0.05$) but was not removed by backward elimination probably to maintain the hierarchy of the model. The three interactions terms included in the model were visualized by contour plots (Figure 3.4). At the same time, the other factors were maintained at their center values

There was a clear linear relation between the enzyme load and the reaction temperature (Figure 3.4a). A yield of 50% was obtained for a high temperature (75°C), high enzyme load (15%) combination, with other factors at their center level. The contour plot showing the interactions between reaction time and substrate molar ratio (Figure 3.4b) indicates that when a high amount of glycerol was present (corresponding with a low substrate ratio), the amount of DAG was low but a longer reaction time will not increase this yield. At lower

glycerol concentrations, an equilibrium was reached after 9 hours, corresponding with ±46% DAG.

Another observation was that up to 9 hours of reaction, a corresponding increase of the temperature resulted in a linear increase for the amount of DAG (Figure 3.4c). The highest enzyme activity (75°C) resulted in a maximum yield after 9 hours of reaction.
To summarize: the above observations (perturbation and contour plots) point out that the maximum yield of 50% DAG was obtained at the highest temperature (75°C), with the highest amount of enzyme (15 wt%), with an intermediate substrate molar ratio (1.12) and a reaction time of 9 hours. Addition of extra water was not necessary.





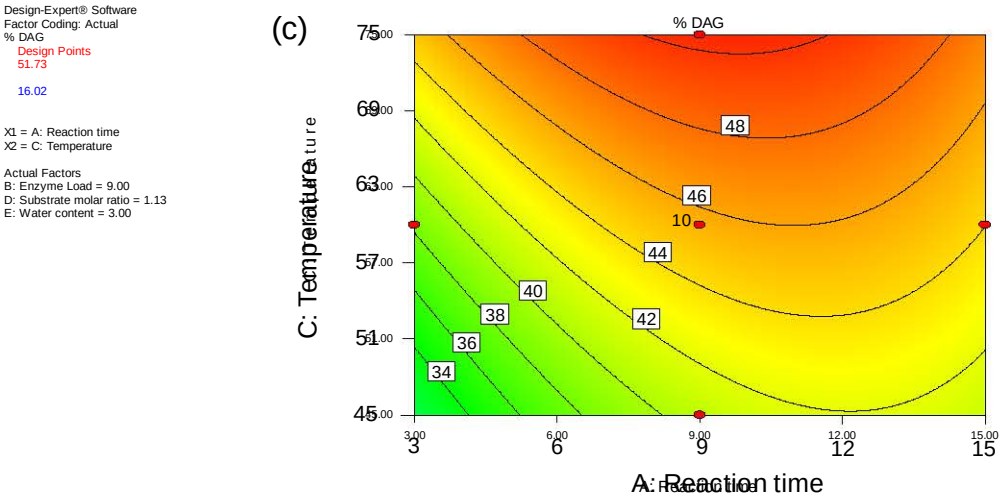


Figure 3.4 Contour plots of the parameters with a significant contribution to the model

The Design Expert software was able to generate the optimal conditions for the highest DAG yield. The following optimal conditions should generate 53% DAG:

- Reaction time: 10.26 hours
- Enzyme load: 15%
- Temperature: 75°C
- Substrate molar ratio: 1.35

The corresponding contour plot is given in Figure 3.5.

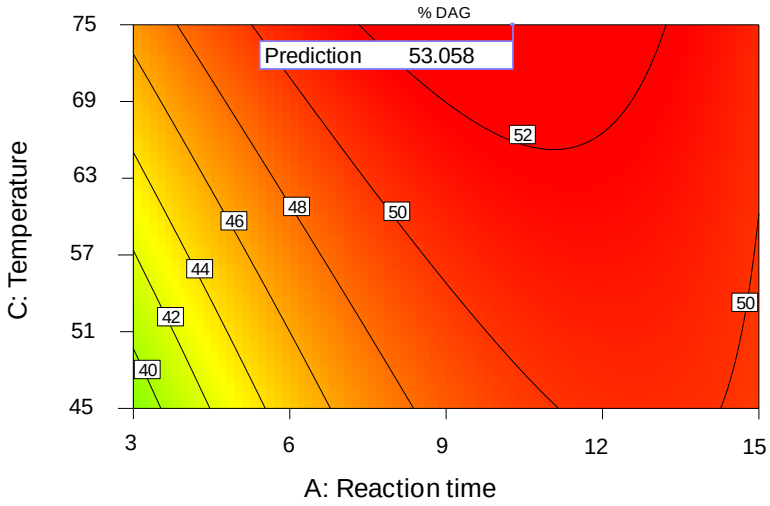


Figure 3.5 Contour plot for optimal factor levels

3.4.1.2.5 Further optimization and model verification

Before scaling up, the information of the response surface modeling was verified by an extra set of experiments as shown in Figure 3.6.

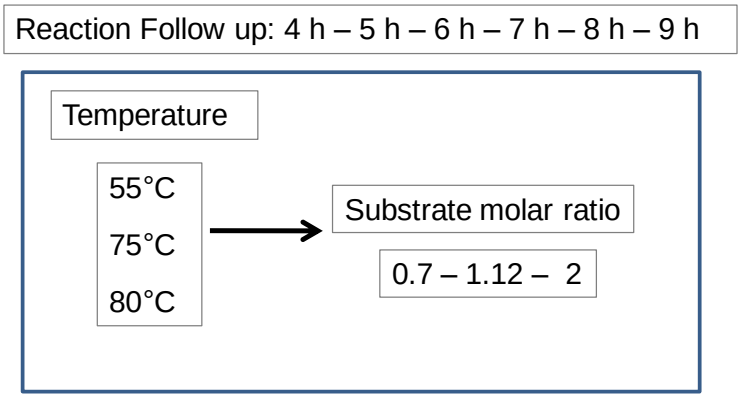


Figure 3.6 Schematic overview of the extra experiments to verify the conclusions of the RSM

Based on the previous experiments, water was no longer added. A higher reaction temperature (80°C) was included to evaluate its effect on DAG yield. The temperature of 55°C was chosen as it was defined as the optimum temperature for the enzyme to operate (Ferreira-Dias et al., 2001). The amount of MAG in the reaction mixture can be advantageous if TAG are simultaneously decreased. As it was not possible to model the %MAG with the response surface methodology, the amount of glycerol was varied to see the impact on the conversion degree of TAG. Different samples were analyzed as function of reaction time. The amount of enzyme was not varied and set at 15% as this parameter had the highest positive influence on the DAG yield in the model.

To assess the effect of the reaction time, the reaction with a substrate molar ratio of 1.12 was taken as a representative sample (Figure 3.7). At 55°C, the reaction was still in progress during the first 6 hours. The amount of TAG was still decreasing at the expense of increasing amounts of MAG and DAG. An equilibrium was attained after 7 hours, yielding 48% DAG. On the contrary, at the higher temperatures (75 and 80°C) a reaction time of 4 hours was sufficient to reach an equilibrium amount of ±50% DAG. Similar trends were observed at the other substrate ratios (results not shown). In contrast with the optimum conditions suggested by the model, a reasonable lower reaction time was sufficient to produce 50% DAG. So the model made an overestimation of the reaction time. Increasing the temperature to 80°C did not show any benefit.

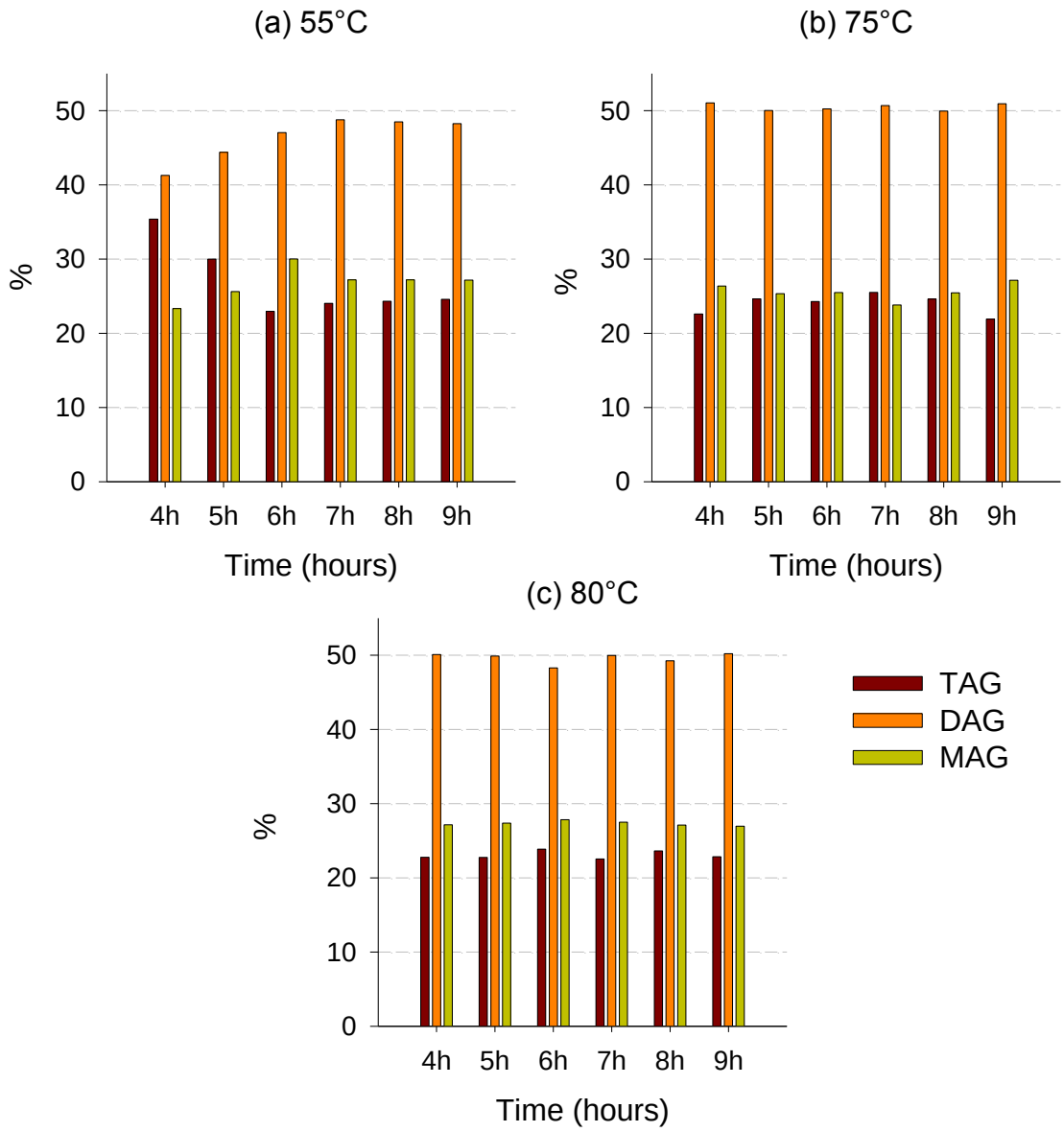


Figure 3.7 Acylglycerol profile as function of time of the enzymatic glycerolysis process, at (a) 55°C, (b) 75°C and (c) 80°C, with substrate molar ratio of 1.12

To evaluate the effect of the different substrate ratios, the different reactions with their corresponding level of MAG, DAG or sum of the previous are presented in Figure 3.8. The substrate ratio mainly had an effect on the amount of MAG: more glycerol resulted in significantly higher amounts of MAG and lower levels of DAG. The remaining TAG were consistently lower, so more TAG converted to partial acylglycerols. Similar trends were observed at the different reaction temperatures. As earlier stated, a higher conversion degree is advantageous for the later separation process. On the other hand, for a large scale production, a lower amount of glycerol is beneficial, as potential problems caused by the high viscosity of glycerol can be avoided (Kristensen et al., 2005b).

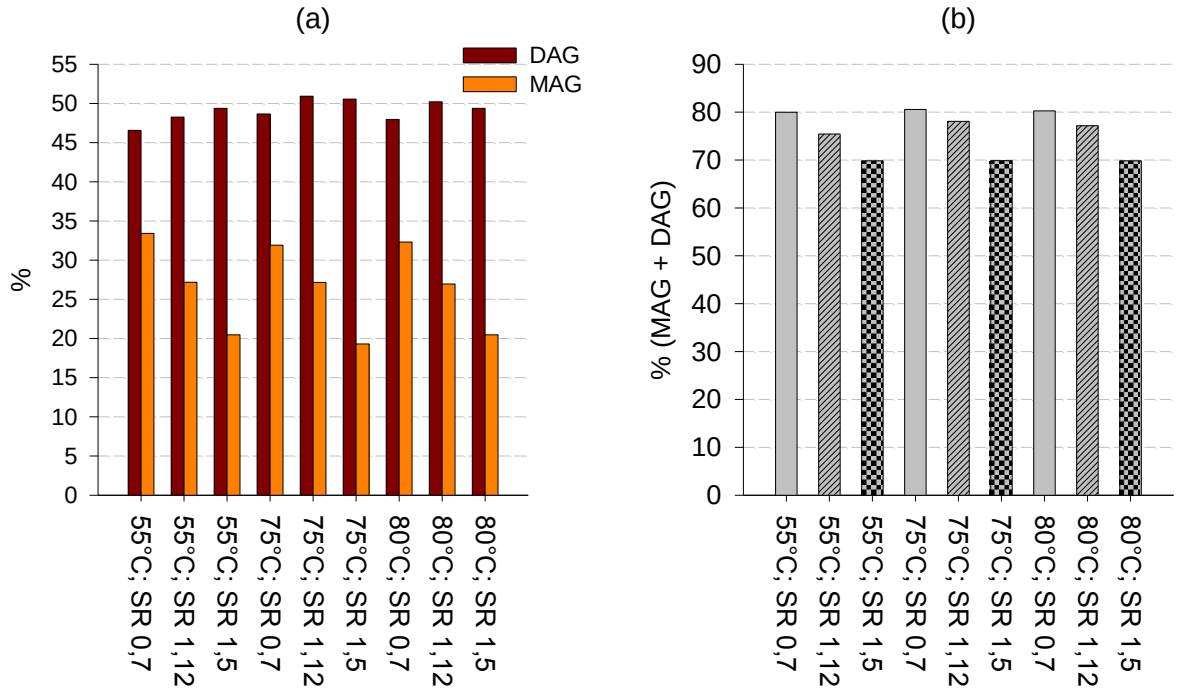


Figure 3.8 Amount of DAG and MAG (a) and total sum (DAG+MAG) (b) for the different reaction conditions after 9 hours reaction

When taking practical considerations into account, following reaction conditions were chosen to obtain an acceptable DAG yield:

- Reaction time: 6 hours, a reasonable time to obtain equilibrium
- Reaction temperature: 70°C average temperature, still in the range of the maximum enzyme activity
- Enzyme load: 15 wt% of the oil mass, a high amount of enzyme didn't demonstrate negative effects
- Substrate molar ratio: 1.12, a combination of a reasonable conversion with a reasonable amount of glycerol

3.4.1.3 Upscaling of the process

In the upscaling to a 0.5 kg glass batch reactor a similar DAG yield of 50% was achieved. A reaction equilibrium was already observed after 4 hours. Further upscaling to a self-made 5 l glass reactor reduced the yield to ±45% DAG and ±30% MAG. This was probably due to a less efficient mechanical stirring. A higher stirring speed was necessary (350 – 400 rpm) to homogenize the mixture in the larger recipient. It is known that lipases may lose some activity especially in batch reactions as the mechanical stirring may crush some of the immobilized lipase particles (Kristensen et al., 2005b). Nevertheless, the amount of DAG was still in the acceptable range to use this reactor for larger scale productions.

3.4.2 Short-path distillation

The DAG rich cocoa butter based product was further purified using short-path distillation (SPD). Table 3.6 gives an overview of the acylglycerol (based on HPLC analysis) and fatty acid composition of the enzymatically produced feedstock for this SPD experiment. Four successive steps, each with specific vacuum, temperature and product flow settings, were necessary to obtain high purity cocoa butter DAG. An overview of the experimental set up is given in Table 3.7. From step II to IV, the temperature was varied to obtain the desired separation.

In the first step a low vacuum was applied at a considerable low temperature and the condenser was set at room temperature to remove the present water. The residue was collected in the cold trap.

Table 3.6 Acylglycerol and fatty acid composition of the feedstock

	Acylglycerol (%)			Fatty acid composition (%)		
	MAG	**DAG**	**TAG**	**C16:0**	**C18:0**	**C18:1**
Feed (Res II)	32.3	45.1	22.7	27.2	35.4	32.3

Table 3.7 Overview of the different step during the short-path distillation

	Distillation parameters			Distillate	Residue
	P (mbar)	Flow rate (g/h)	Temp (°C)		
Step I	25	1500	120	Water	FFA, Glyc, MAG, DAG, TAG
Step II	1	700	180-200-220	FFA, Glyc	MAG, DAG, TAG
Step III	0.1	390	180-190-200-210-220-230	MAG	DAG, TAG
Step IV	0.0005	300	245-250-255-260-270-280	DAG	TAG

3.4.2.1 Step II: Removal of the FFA

In step II, the vacuum was set at 1 mbar, flow rate at 700 kg/h and three temperature settings were investigated. During the glycerolysis reaction around 4% free fatty acid were formed (Table 3.8). During the distillation, FFA and glycerol were condensed and collected as distillate. An increased evaporator temperature, decreased the amount of FFA in the residue and the distillate yield increased. Acylglycerol analysis confirmed that the higher yield at 220°C was due to distillation of MAG. Even at 200°C, some MAG were already

detected in the distillate. At 180°C, the remaining amount of FFA was still too high, therefore 200°C was selected to remove the glycerol and FFA. The fatty acid composition of the feed of the first step was very similar to the composition of the residue.

Table 3.8 Amount of free fatty acids (FFA) and %Distillate in the feed and residue as function of evaporator temperature

	FFA (% oleic acid)	% Distillate
Feed	3.84	/
Residu 180°C	1.0	3.8
Residu 200°C	0.5	5.3
Residu 220°C	0.2	10.3

3.4.2.2 Step III: Removal of the MAG

In step III, a vacuum of 0.1 mbar was applied and the evaporator temperature was varied between 180°C to 230°C in steps of 10°C. Figure 3.9 shows the acylglycerol profile as function of the evaporator temperature and Table 3.9 lists the corresponding distillate yield. The fatty acid distribution of the three main fatty acids (palmitic acid, C16:0; stearic acid, C18:0; oleic acid, C18:1) is also presented in this table. Up 200°C, there was a steep increase in the distillate yield corresponding with an decreased amount of MAG in the residue. The yield still increased up to 31% at a temperature > 200°C. At 220°C, a small decrease in the amount of DAG was observed indicating that some of the DAG were also distilled off at these higher temperatures. Around 1% DAG was detected in the corresponding distillate.

The fatty acid composition of the distillate was a measure of the MAG formed during the enzymatic reaction. The amount of palmitic acid was reduced as function of time indicating that at lower temperatures, monopalmitine was preferably distilled off. As temperature increased the fatty acid composition was similar to the one of the feed. The residue of this third step (Res III) had a slightly lower amount of palmitic acid. As the used enzyme Novozym 435 is a non-specific enzyme with no preference towards a specific fatty acid, only small differences were expected. To be sure that all the MAG were removed, it was decided to choose 220°C as the optimum temperature for this step. A lower temperature (150 – 175°C) can be applied at higher vacuum conditions (0.001 – 0.0005 mbar) (Kristensen et al., 2005b; Lin and Yoo, 2009; Yang et al., 2004).

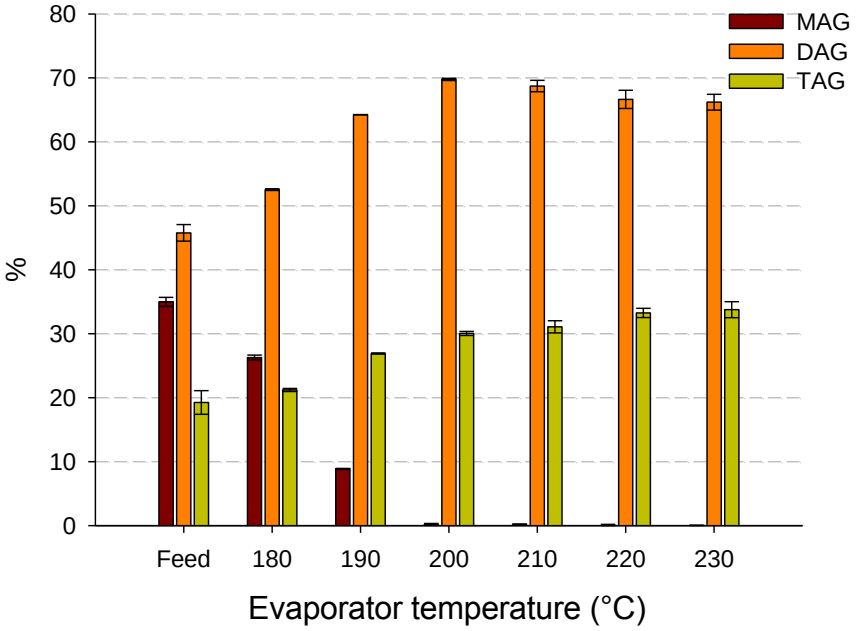


Figure 3.9 Acylglycerol profiles of the residues as function of evaporator temperature

Table 3.9 The yield and fatty acid composition of the distillate as function of evaporator temperature; fatty acid composition of residue II and residue III

		Fatty acid composition (%)		
	% Distillate	C16:0	C18:0	C18:1
Feed (Res II)	/	27.1	35.5	32.6
180°C	11.4	39.1	29.4	27.4
190°C	18.8	34.5	32.1	29.6
200°C	26.3	29.3	35.2	31.2
210°C	28.7	27.8	36.4	31.3
220°C	29.7	27.0	36.5	31.5
230°C	31.2	28.2	35.9	31.0
Res III (220°C)	/	26.6	35.1	33.1

3.4.2.3 Step IV: Purification of the DAG

After three sequential distillation steps, the residue mainly contained DAG and TAG, with a ratio 70/30. A very high vacuum of 0.0005 mbar was applied and the feed rate was slightly decreased (Table 3.7). At 245°C, the distillate contained 98% DAG but around 15% DAG remained in the residue (Figure 3.10). The higher the evaporator temperature, the lower the residual amount in the residue. On the other hand, more entrainment of the TAG into the distillate occurred. An increasing distillate yield was observed with increasing temperature.

Up to 255°C, the amount of palmitic acid was significantly higher, giving evidence that at the lower temperatures components with a lower molecular weight and boiling point were preferentially distilled off. At 260°C, the fatty acid composition of the residue showed the characteristic fatty acid composition of cocoa butter (Table 3.6). At lower or higher temperatures, the amount of palmitic acid was lower, illustrating the preferential distillation of a more palmitic based DAG or entrainment of more palmitic based TAG respectively. Based on these findings, 260°C was selected as the most suitable temperature for this fourth step. For hydrolyzed soybean oil lower temperatures (230 – 240°C) gave acceptable results at similar vacuum conditions (Wang et al., 2010). Kristensen (2005b) reported similar temperatures (250 – 265°C) for the SPD of sunflower and rapeseed oil based DAG.

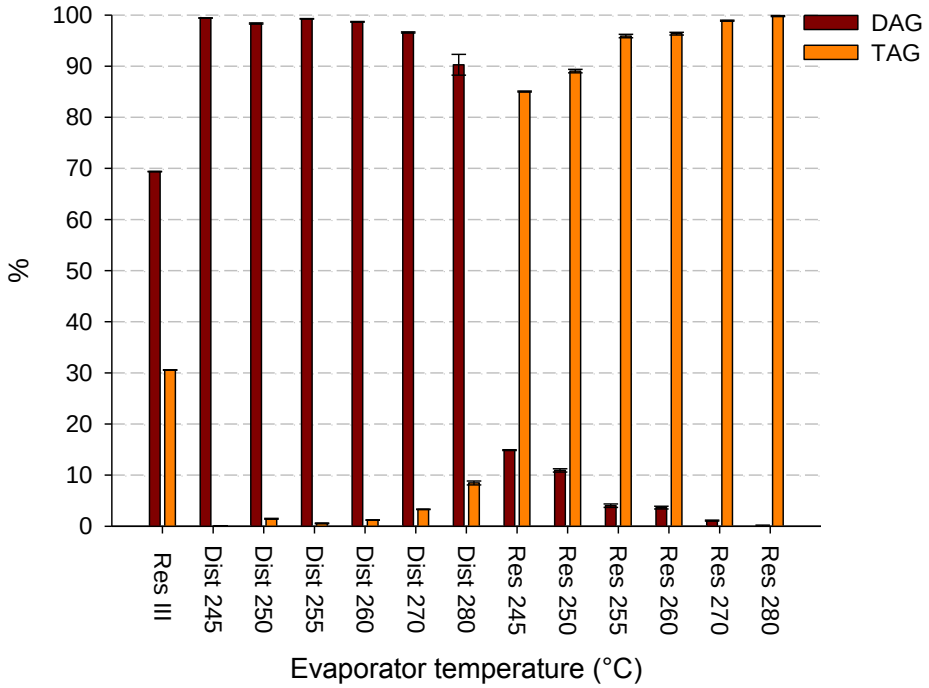


Figure 3.10 Acylglycerol profiles of the distillate and residue as function of evaporator temperature

Table 3.10 Distillate yield and fatty acid composition of the distillate as function of evaporator temperature

		Fatty acid composition (%)		
	% Distillate	C16:0	C18:0	C18:1
245°C	48.0	30.2	34.4	33.8
250°C	52.7	29.0	33.8	32.6
255°C	61.5	28.0	34.4	33.0
260°C	61.6	27.6	34.4	33.4
270°C	68.0	27.2	34.6	32.6
280°C	74.7	27.3	34.9	33.3
Res IV (260°C)	/	27.9	35.2	31.4

3.4.2.4 Process overview

Based on the discussion above, the optimum conditions for the SPD process were selected and they are listed in Table 3.11.

A mass balance (based on 1 kg feed) was set up and outlined in Figure 3.11. In the first two steps 7% of by products (water, FFA, glycerol) were removed. Steps III and IV aimed at separating the different acylglycerols. On acylglycerols base, 31% MAG and 42% DAG were obtained. Both distillates contained 1 to 2% DAG and TAG respectively due to entrainment. The process efficiency (based on the values of Table 3.6) was then ~96% for the MAG and ~93% for the DAG.

Table 3.11 Optimum condition for short-path distillation

	Distillation parameters		**Distillate**	**Residue**
	P (mbar)	Temp (°C)		
Step I	25	120	Water	FFA, Glycerol, MAG, DAG, TAG
Step II	1	200	FFA, Glycerol	MAG, DAG, TAG
Step III	0.1	220	MAG	DAG, TAG
Step IV	0.00001	260	DAG	TAG

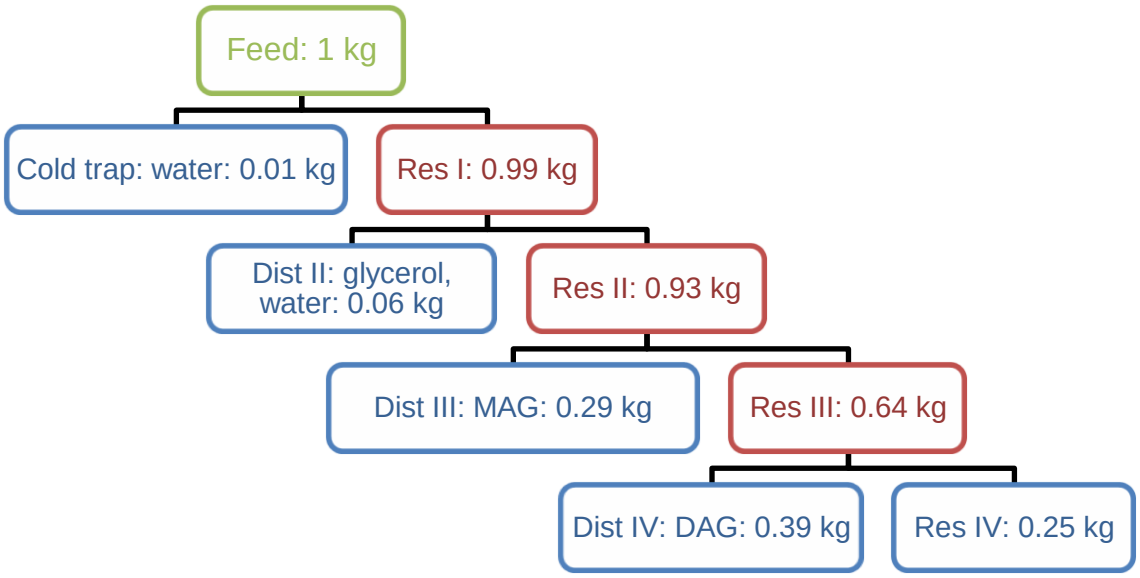


Figure 3.11 Mass balance optimized short-path distillation

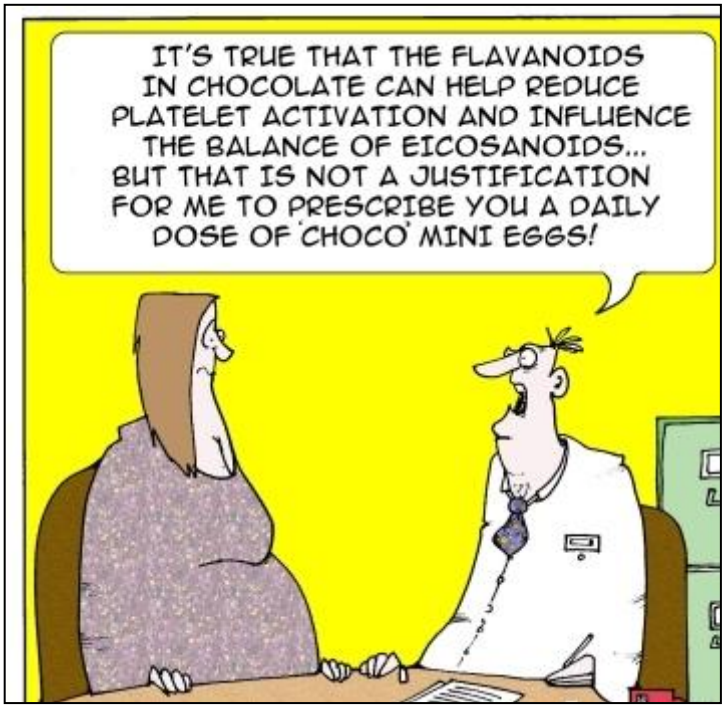
3.5 Conclusions

In this chapter, the objective was to develop a process for the production of DAG originating from cocoa butter through enzymatic glycerolysis and short-path distillation. Five reaction parameters of the enzymatic glycerolysis reaction were optimized using response surface methodology. In this work a fractional face centered central composite design was used as a tool to identify the most important reactions settings. Evaluation of the resulting model provided insight in how process conditions can be varied to optimize the DAG yield. However, an extra set of experiments was necessary to justify the model. Based on these experiment, following optimal conditions were selected: no water addition, 6 hours at 70°C, with addition of 15 wt% enzyme and a substrate molar ratio of 1.12.

In the last step of the production process, the most suitable pressure-temperature combinations of the four-step short path distillation process were defined. In the last distillation step, it was possible to obtain a highly purified DAG fraction at a very low vacuum and a temperature of 260°C, with an acceptable process efficiency of 93%.

Chapter 4

Influence of cocoa butter diacylglycerols on the phase behaviour of cocoa butter



4.1 Introduction

As described in section 3.1.2, nutritional studies have indicated that mainly the 1,3-DAG possess some nutritional benefits compared to the TAG. Moreover, more than 40% DAG are required to achieve its beneficial health effects. However, very little information has so far been published concerning the influence of high concentrations of DAG on physical properties of TAG (Cheong et al., 2009b; Saberi et al., 2011a).

Saberi et al. (2011b) investigated the influence of low and high concentrations of enzymatically synthesized palm based DAG and concluded that the addition of 30 and 50% significantly increased nucleation, crystallization rate and changed the crystal growth mode. This may be interesting from a nutritional point of view but also of technical interest to inhibit for example post hardening in palm–based refrigerated margarines. A combination of the palm based DAG with sunflower and palm kernel oil (50/35/15 w/w) was shown to be successful in a soft tub margarine formulation. The nutritional benefits of the DAG were combined with a lower amount of saturated fatty acids (Saberi et al., 2011a).

In the study of Cheong et al. (2009b), the effect of lard partial acylglycerols on the melting and crystallization in blends with rapeseed oil was investigated. The presence of a low concentration of lard-DAG was found to significantly delay nucleation and crystal growth velocity of the blends. Meanwhile, a high concentration of lard-DAG was found to promote nucleation and crystal growth.

4.2 Research strategy

Previous studies have characterized DAG originating from milk fat (Yang et al., 2004), lard (Cheong et al., 2009b), palm oil and its fractions (Cheong et al., 2007; Cheong et al., 2009a, 2010; Saberi et al., 2011a; Saberi et al., 2011b) but to the best of our knowledge, no study so far that has characterized enzymatically produced DAG originating from CB. Consequently, the first goal of this research was to characterize the physicochemical properties of the produced CB DAG. The different DAG species formed during the enzymatic reaction were identified and quantified. In the next step CB was blended with the CB based DAG to screen their applicability in confectionary products. The non-isothermal crystallization and melting behaviour was studied by DSC, pNMR was used to determine the solid fat content as function of temperature. The influence of the different DAG concentrations on the microstructure was visualized by PLM.

4.3 Materials and methods

4.3.1 Samples

The CB DAG were melted at 80°C and added to cocoa butter (Barry Callebaut, Wieze) in concentrations between 0 – 100% (w/w) with 10% increments.

4.3.2 Acylglycerol analysis by HPLC

The method is described in detail in 3.3.7.

4.3.3 Differential scanning calorimetry (DSC)

The principle of the DSC analysis is explained in section 2.3.4.3.
To define the *non isothermal melting and crystallization behaviour*, following time-temperature program was applied: equilibrate at 80°C for 10 min to ensure a completely liquid state and erase the crystallization memory of the sample. Samples were subsequently cooled at 5°C/min to -80°C to crystallize the sample. After a short isothermal period the sample was heated again at 5°C/min to 80°C. Each analysis was executed in triplicate.

4.3.4 Solid fat content

The Solid Fat Content (SFC) is generally measured by pulsed magnetic resonance (pNMR). A more detailed description of this method is given by Vereecken (2010). In this research a Maran ultra pulsed field gradient NMR (Oxford Instruments, Tubney Woods, Abingdon, UK) was used to perform the analyses.

For confectionary fats, a tempering step of 40 hours at 26°C is used to ensure that cocoa butter and similar SOS-type (S= saturated, O: oleic acid) of fats are converted to their β polymorph before measurement. The fat was melted and 3.5 ml was placed in NMR tubes (3 replicates) and held at 70°C for 30 min to erase thermal history. Then, they were submitted to the tempering treatment of the IUPAC 2.150 serial tempered method. The SFC was determined in the range of 5 – 40°C at 5°C intervals following 60 min incubation in a thermostatic water bath.

4.3.5 Determination of the microstructure by polarized light microscopy

The microstructure was evaluated by using polarized light microscopy (PLM). The microscopic analyses were performed with a Leitz Diaplan microscope (Pleitz, Wetzlar,

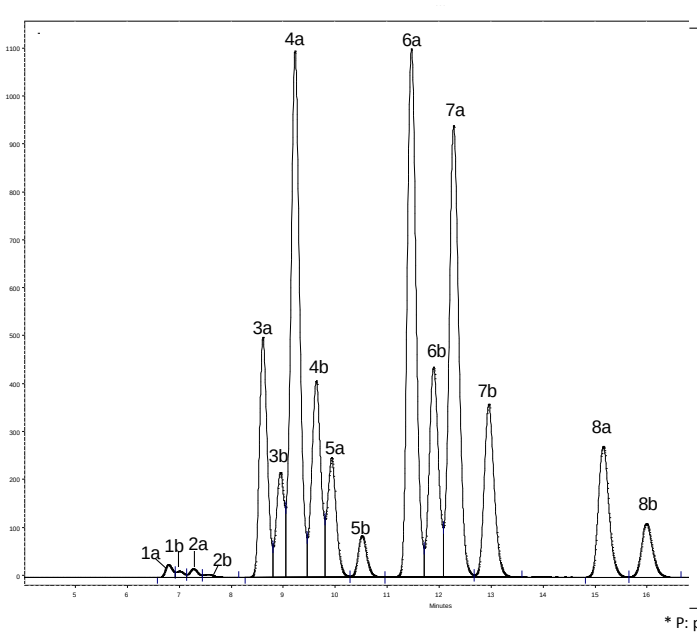
Germany) equipped with a Linkam temperature controlled carrier plate and a Linkam TMS 91 controller (Linkam, Surrey, United Kingdom). A droplet of the liquid oil was applied to the carrier glass and covered with a cover slide. The sample was subsequently heated to 70°C and kept at this temperature for 10 min. In the next step, the sample was cooled at 10°C/min to the selected crystallization temperature and held there for a predefined time. Images were acquired meanwhile using an Olympus color view camera (Olympus, Aartselaar, Belgium).

4.4 Results and discussion

4.4.1 Characterization of the substrates

The TAG composition of the used CB is given in Annex III. This cocoa butter was also used for the enzymatic glycerolysis reaction to produce the DAG. The CB contained typical amounts of the three major TAG: POP (18.09%), POSt (39.90%) and StOSt (27.88%).

The DAG fraction was characterized by HPLC ELSD method as described in 3.3.7. A typical chromatogram is given in Figure 4.1. It can be seen that not all the peaks were baseline separated so the values are indicative. The final ratio 1,3-DAG/1,2-DAG was ~7/3, a typical ratio for equilibrium conditions. There are three predominant species (including both isomers): palmitoyl-oleolyl-glycerol, PO (~25%), stearoyl-oleolyl-glycerol StO (~25%) and palmitoyl-stearoyl-glycerol PSt (~24%). The DAG compositions were further categorized (including 1,3 and 1,2 isomers) into disaturated (SS), monounsaturated (SU) and diunsaturated (UU) DAG. The monounsaturated fraction represents more than half of the present DAG.



Peak Number	DAG*	%
1a	1,3-LO	0.37
1b	1,2-LO	0.18
2a	1,3-PL	0.27
2b	1,2-PL	0.13
3a	1,3-OO	7.33
3b	1,2-OO	3.19
4a	1,3-PO	18.28
4b	1,2-PO	6.99
5a	1,3-PP	4.13
5b	1,2-PP	1.48
6a	1,3-StO	18.1
6b	1,2-StO	7.60
7a	1,3-PSt	17.15
7b	1,2-PSt	6.75
8a	1,3-StSt	5.71
8b	1,2-StSt	2.34
	SS	37.56
	US	51.37
	UU	11.07

* P: palmitic; St: stearic; O: oleic; L: linoleic acid; S: saturated; U: unsaturated

Figure 4.1 DAG profile of cocoa butter based DAG

The non-isothermal melting and crystallization thermogram of the CB based DAG is shown in Figure 4.2.Three main melting areas can be defined. A first small endothermic peak was determined between 5 and 20°C, corresponding with the melting of the unsaturated DAG, representing 11% of the total amount of DAG. The second peak is observed between 20 and 40°C with at peak maximum of 33°C. The components of this melting fraction will be related to the SU DAG. Little information is available about the melting behaviour of the present SU DAG. Recently Craven and Lencki (2011) reported a melting point of 42°C for 1,3-PO. Bailey (1950) reported 54°C for SO and 46°C for PO but the isomeric form was not identified so these values are only indicative. Moreover, as illustrated in Table 3.1, the difference in melting point between the 1,3-DAG and 1,2-DAG can be quite high. So it is plausible that some of SU components have co-crystallized with the SS components of the high-melting peak between 40°C and 70°C.

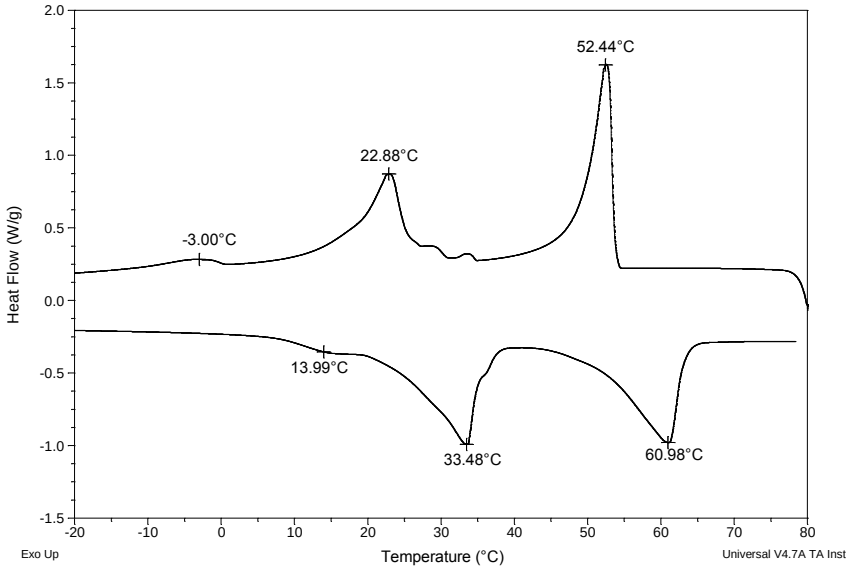


Figure 4.2 Non isothermal melting thermogram of the CB based DAG

4.4.2 Phase behaviour of CB-CB DAG blends

Cocoa butter was blended with the CB DAG and phase behaviour was described by using DSC, pNMR and the microstructure was visualized by PLM.

4.4.2.1 Crystallization and melting behaviour as measured by DSC

The prepared blends were submitted to a time-temperature profile and phase changes were recorded by DSC. The crystallization and melting behaviour was recorded by applying the method described in section 4.3.3. As the samples were rapidly cooled they crystallized in the unstable polymorphic form. As the CB DAG were a mixture of two isomeric forms and

additionally these isomers show different polymorphic behaviour (section 3.1.1), it is difficult to predict in which polymorphic form they will crystallize. Figure 4.3 shows the crystallization and melting thermograms of CB and its blends with the CB DAG.

As stated by Tan and Man (2000) the DSC crystallization curve, which is only influenced by the chemical composition of the sample, and not by the initial crystalline state, is more reproducible and simpler than the melting curve. Indeed, in this study the melting thermograms in Figure 4-3b showed more complex behaviour with a higher number of peaks and shoulders, in particular when the amount of DAG increased.

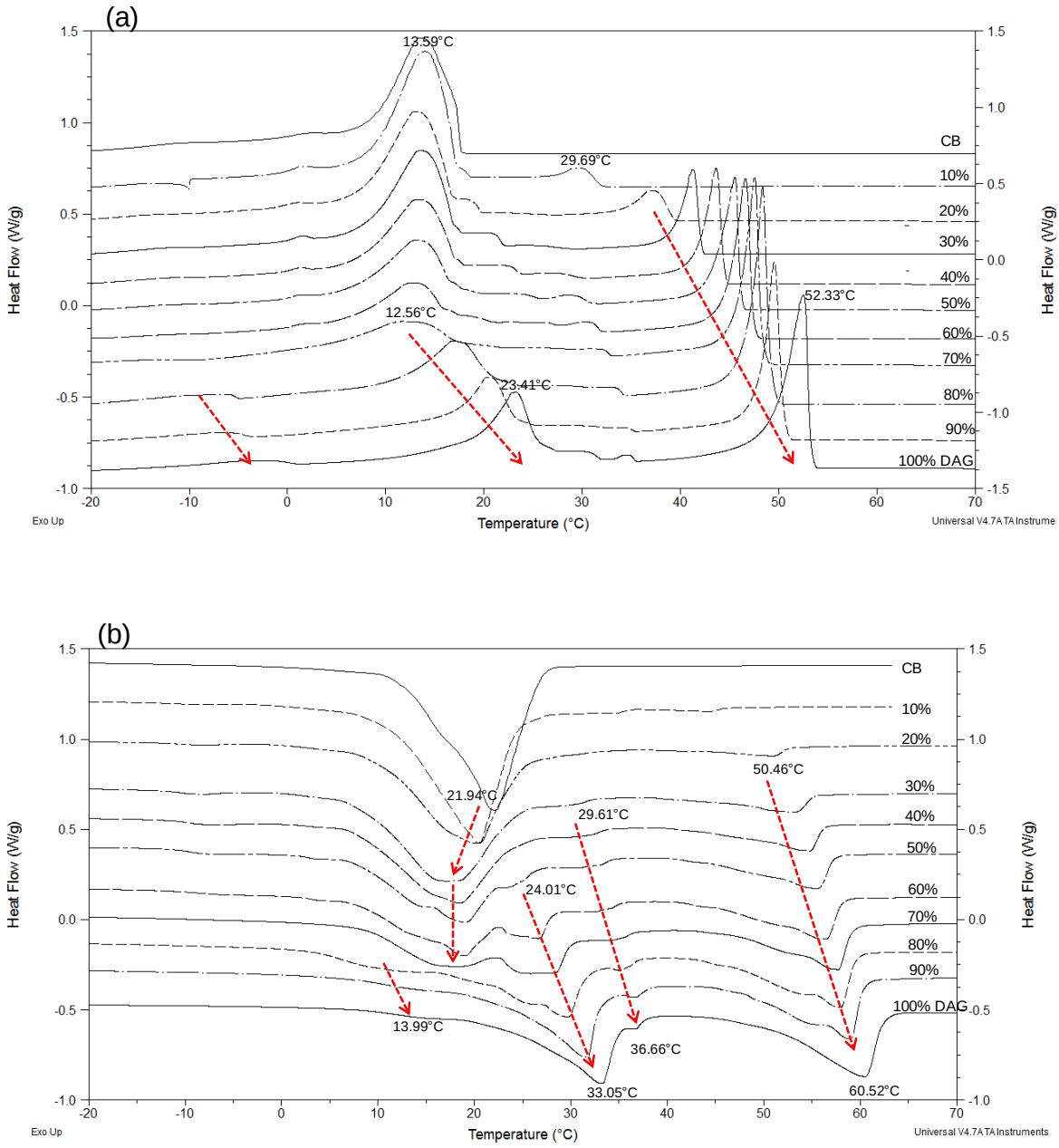


Figure 4.3 (a) Crystallization and (b) melting thermograms of CB – CB DAG blends from 0 to 100% with 10% interval

As CB only showed one major exothermic peak, adding 10% DAG introduced a second exothermic peak at a higher temperature. Further addition of DAG resulted in a proportional increase of the intensity of this peak with a shift to higher temperatures. This was undoubtedly related to the crystallization of the high-melting DAG fraction, i.e. the SS DAG. Consequently, the crystallization onset temperature (determined by the intersection of the baseline with the absolute highest tangent of the crystallization curve) increased as function of DAG increment as exemplified in Figure 4.4. CB had a T_{onset} of 17°C, in correspondence with Campos et al. (2010).
The intensity of crystallization peak related to the CB TAG (in the case of pure CB) decreased and a shoulder emerged at the right side. This pointed out that to a certain extent the low-melting DAG (UU and SS) are intersoluble and/or can co-crystallize with the CB TAG. Beyond 70% DAG, this peak moved to higher temperatures to end up with a peak maximum around 23°C for the pure DAG.

The melting endotherm (Figure 4.3b) of CB shifted to lower melting temperatures up to 30% DAG addition. This effect of melting point depression was attributed by Siew and Ng (2000) to the formation of eutectic mixtures between DAG and TAG.
At 30% DAG a shoulder appeared around 29.6°C. At 50% replacement another shoulder (~24°C) emerged from the main low-melting peak but at 80% DAG this peak became dominant and on his turn dissolved the lower melting peak (observations are indicated with red arrows). The high-melting peak, related to the high-melting DAG fraction shifted to higher temperatures proportional with the amount of DAG. Simultaneously, the intensity of this peak increased.

Figure 4.4 shows the evolution of the crystallization heat as function of DAG concentration. Compared to CB, the crystallization enthalpy of the CB DAG almost doubled. This may indicate that DAG crystallize in a denser crystal packing. Knowledge about the crystallization or melting enthalpy is important from an engineering point of view. Heat transfer can be important in crystallizers, or scraped surface heat exchangers.
On the other hand, this can also be important for product functionality. The typical TAG composition of CB melts over a narrow range around body temperature. This characteristic melting profile is responsible for an intense flavour release and a cooling sensation in the mouth (Smith, 2001). As DAG require more energy to melt them, they could assess a natural cooling sensation, not necessarily over a narrow melting range, when applied in fat rich products.
If TAG and DAG do not interact during blending, the blends should be located on a straight line that connects the outer points, as indicated in Figure 4.4. It can be seen that up to 60%

DAG, the crystallization enthalpy was lower than expected so this may indicate that the DAG interact with the TAG crystal network. In a blend with 70% or more DAG, they behave independently as their enthalpy is located on the straight line.

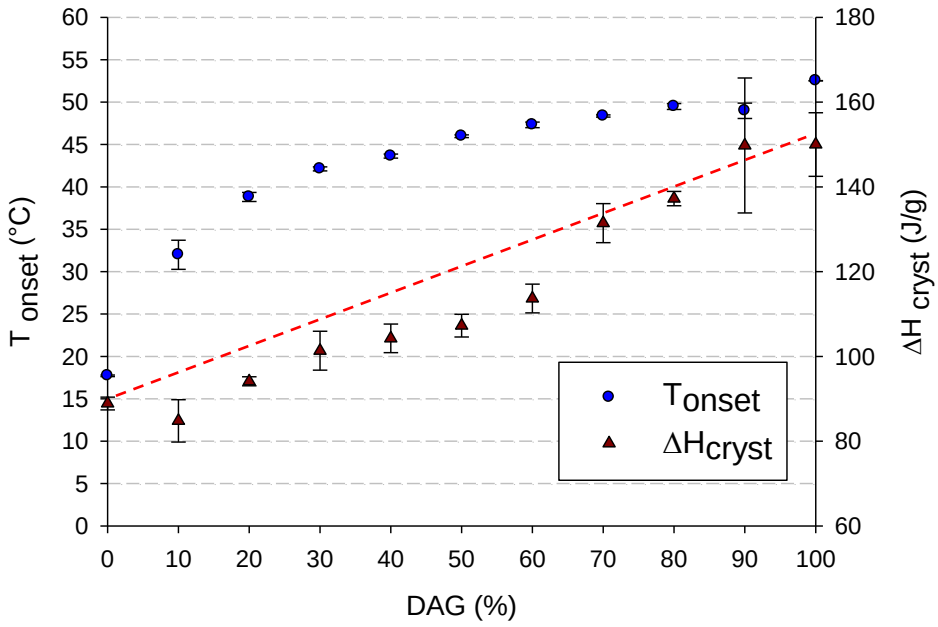


Figure 4.4 Crystallization onset and heat of crystallization for the blend CB with CB DAG

4.4.2.2 Solid fat content as measured by pNMR

As CB is considered as a stabilizing fat, the blends were subjected to the official IUPAC tempering procedure, 40 hours at 26°C prior to recording the SFC profile as function of temperature. The results of the measurements are presented in Figure 4.5 and to improve the readability and the interpretation, the results were presented in two curves. CB had a high SFC up to 20°C, followed by a steep decrease between 25 and 35°C and no solid fat was detected above body temperature.

When up to 50% of the CB was replaced by the DAG, the SFC in the temperature range 15°C to 25°C, related to the hardness of a fat, gradually decreased but when more DAG were introduced (Figure 4.5b) the SFC increased again. Moreover, the solid fat profile of CB and the 100% DAG overlap up to a temperature of 25°C. All the blends, except 90% or more, had a lower heat resistance (SFC between 25 – 30°C) compared to CB. All the blends showed a residual amount of solid fat above body temperature which would cause a waxy mouth feel, limiting their applicability in confectionary products. This finding is attributed to the presence of the high-melting DAG, the SS-group.

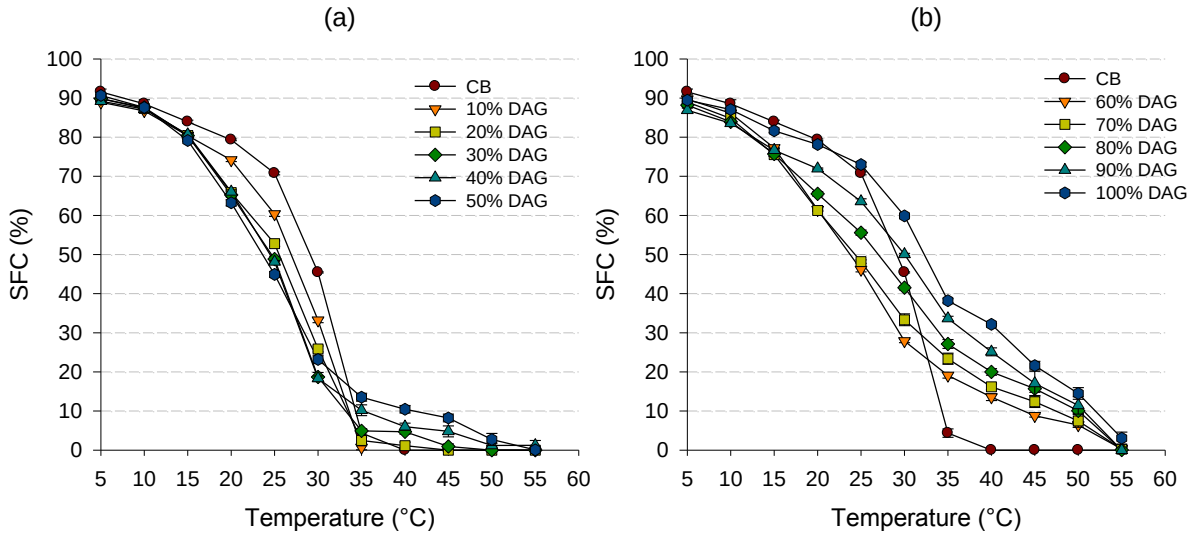


Figure 4.5 Solid fat content for (a) CB – CB DAG blends up to 50% (b) CB – CB DAG blends up to 100%

Phase behaviour can be explained in terms of solution behaviour using an isosolid diagram. Figure 4.6 shows the isosolid diagram constructed based on the SFC data. In this composition-temperature graph, the solid lines connect those points with the same SFC value. If the lines are parallel and approximately horizontal, then the components exhibit good compatibility meaning that they give complete miscibility in the solid and liquid phases. If this is not the case, with the extreme case occurring when eutectic mixtures are formed (minimum points), softening occurs (Solis-Fuentes and Duran-de-Bazua, 2004; Timms, 2003). The isosolid lines of cocoa butter are close together in a narrow temperature range as a result of its narrow melting profile. If the contribution of the DAG increased, the isosolid lines diverged over a broader temperature range. Above 50% DAG the isosolid lines linearly increased indicating that the blends required a higher temperature than CB to completely melt. Above 30% SFC, the isosolid lines tend to show a minimum in the concentration range 20 – 60 % DAG indicating the appearance of eutectic behaviour.

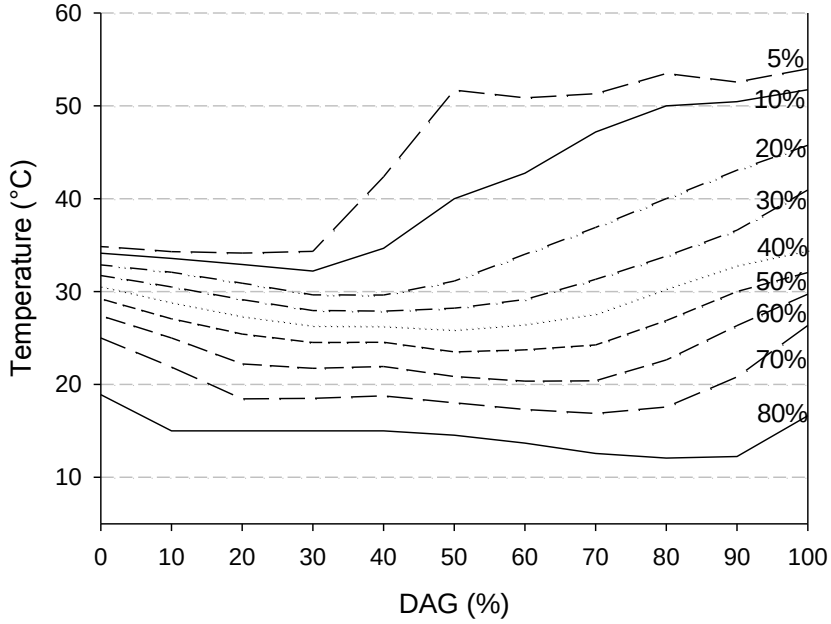


Figure 4.6 Isosolid diagram of mixtures of CB with CB DAG

4.4.3 Isothermal crystallization as visualized by PLM

During crystallization, fats such as cocoa butter form a three-dimensional network with distinct levels of structure. When acylglycerols are supercooled, they will undergo a liquid-solid transition to form primary crystals with characteristic polymorphism. These crystals then aggregate via a heat- and mass transfer limited process into larger crystal aggregates which on their turn grow further to form a three-dimensional network (Brunello et al., 2003). This can be visualized by PLM. The study of the microstructure of fat is important since many macroscopic properties depend on their crystal network structure (Marangoni and McGauley, 2003). The development of the microstructure and thus the crystallization is strongly related to the chemical composition of the fat or oil. Therefore, CB, samples with 10%, 30%, 50%, 70% DAG and the pure CB DAG were further investigated to visualize the effect of DAG on crystallization at 20°C. Samples were submitted to a time-temperature procedure: the rest plate with the droplet of oil was heated to 80°C to erase crystal memory and then cooled at 10°C/min to 20°C. The formation of the first crystallites was monitored and the microstructural development was recorded at regular base during five weeks. Some selected pictures for the different blends are shown in Figure 4.7. Under polarized light, crystals appear as white spots and liquid fat remains black.

The first column shows the pictures after 10 minutes at 20°C. Crystallization of the CB started 2 minutes after attaining 20°C. For all the other samples crystals were already formed during the cooling phase. For all the samples, the amount of crystals quickly

increased to form a dense network. CB and 10% showed a granular structure but after 10 min the network of the latter seemed more saturated than that of CB. An amorphous mass of very tiny crystals is typical for the α form (Himawan et al., 2006). For 30% DAG and more, spherulitic structures, typical for the β' form (Himawan et al., 2006) were observed during the cooling phase. These spherulites further aggegrated during the first 10 minutes. This aggregation was still visible for 30% and 50% while for the higher concentrations, the spherulites already formed a very dense network. As function of time, the pictures of the samples with 30% DAG or more showed that the network became denser but no large microstructures were formed compared to pure CB and 10% addition. As described by Marangoni and McGauley (2003), the formation of the larger microstructures in CB are due to the phase transition from the β' to the β form. The appareance of these larger structures was different for these two sampes. After two weeks, the CB showed a flowery type of structure with a small granular center and some featherlike leaves. These structures continued to grow during storage and the featherlike structures became predominant. For the CB enriched with 10% DAG, circular microstructures with a larger granular center and smaller featherlike structures on the periphery were pictured. Within the granular center, spherulites with featherlike structure increased in size during further storage at 20°C.

Based on the PLM observations, it can be concluded that microstructure was significantly changed when CB DAG were added to CB.

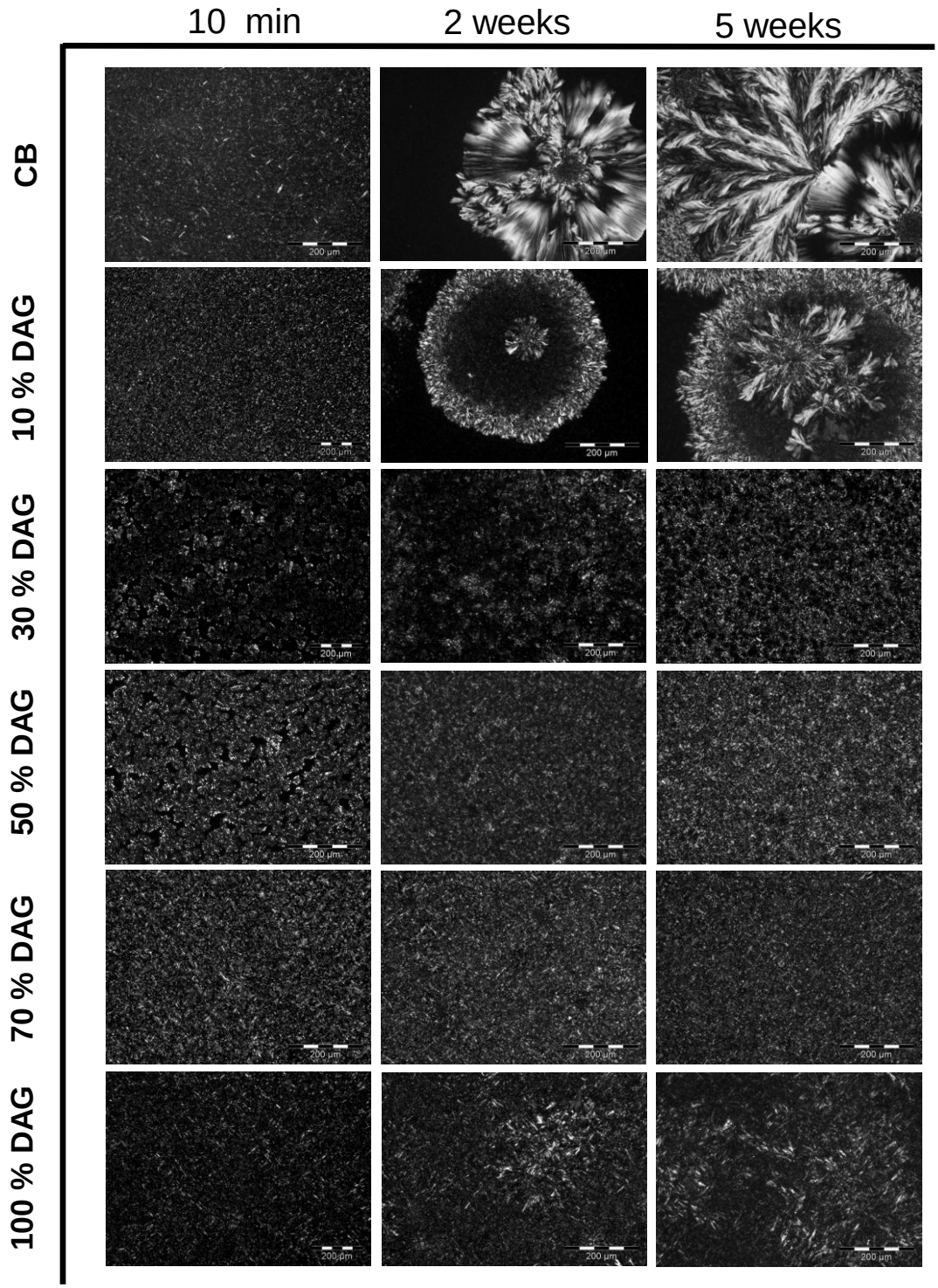


Figure 4.7 Polarized light micrographs of CB – CB DAG blends crystallized isothermally at 20°C

4.5 Conclusions

The enzymatically synthesized CB based DAG contained around 50% monounsaturated DAG, 37% disaturated and 11% diunsaturated DAG. The final ratio 1,3-DAG/1,2-DAG was ~7/3, a typical ratio for equilibrium conditions. Co-crystallization and intersolubility of these DAG resulted three main melting areas. A small endothermic peak was observed between 5 and 20°C; corresponding with the melting of the unsaturated DAG. The disaturated DAG contributed to the high-melting peak observed between 40 – 70 °C. The monounsaturated DAG were probably divided over the latter and the other major endothermic peak between 20 – 40°C.

The CB DAG were blended with CB at varying concentrations between 0 – 100% (w/w) in 10% increments. The onset of crystallization increased with increasing amount of DAG due to the presence of the high-melting fraction. The characteristic melting/crystallization peak of CB, gradually changed upon addition of DAG. The profiles gradually became more complex, probably due to co-crystallization and intersolubility.

In the isosolid diagram, illustrating the phase behaviour of the blends, the isosolid lines showed an eutectic minimum in the region of 20-60% DAG. This indicates a softening effect of the DAG. The crystallization enthalpies also revealed that interactions between the acylglycerols occurred. A major disadvantage was their high amount of residual solid fat above body temperature which would result in a waxy mouth feel during consumption, thus limiting their applicability. Fractionating the DAG to remove the high-melting fraction could therefore be of interest to overcome this problem. The 1,3-DAG have nutritional benefits. Therefore, the lower melting fraction of the CB DAG may offer possibilities to replace a part of the cocoa butter to formulate a healthier chocolate.

However, the macroscopic properties will be affected as the fat crystal network was changed when DAG were added. Visualization of this microstructure showed that for the CB sample large microstructures, indicative of the β_V polymorph developed during storage. At 10% DAG, these large aggregates were still present but the morphology was already considerably changed. At 30% or more, a dense network of spherulites was formed at the beginning of the crystallization period, but upon further storage no large morphological changes were observed.

Chapter 5

Influence of cocoa butter diacylglycerols on the isothermal crystallization of cocoa butter

Chocolate provides instant enjoyment and pleasure, and it is also considered to be one of the favorite foods which are consumed during comfort eating, as well as being the most craved food.

Based on current data, intake of adequate quantities of cocoa and chocolate very likely conveys positive consequences on human health.

Visioli et al., 2009, Critical reviews in Food Science and Nutrition, 49, 299 – 312

5.1 Introduction

The crystallization of TAG mixtures depends on the nature, chain length and saturation/unsaturation characteristics of the TAG and the interaction of these TAG with each other. Partial glycerides, FFA, phospholipids as minor constituents have significant effects on the physicochemical properties of fats and oils. These minor components can be present indigenously or can be added.

As DAG have a free hydroxyl group, they have a slightly lower hydrophobicity compared to TAG and this may account for their physicochemical properties (e.g. melting point, interfacial chemical properties etc.) that are different from the TAG (Yasukawa and Katsuragi, 2004). As several studies have suggested the effect of minor amount of DAG on the crystallization of fats and oils, an overview will be given of the state-of-the art literature related to this subject.

5.1.1 Effects of DAG on crystallization behaviour

When fats, containing TAG, minor components or additives, are cooled from the melt to a temperature below their melting point, they undergo a liquid-solid transformation to form primary crystals with a specific shape and size. This is the nucleation step. The primary crystals grow into larger microstructural elements (~6 μm) which then aggregate via a mass- and heat transfer limited process into larger microstructures (~100 μm). The aggregation process continues until a three dimensional network is formed by the collection of microstructures. Trapped within this solid network structure is the liquid phase of the fat (Narine and Marangoni, 1999). Minor components may influence the different crystallization steps. A schematic overview proposed by Smith et al. (2011), defines the different modes of action of additives (Figure 5.1). They can interact on the nucleation level, influence the crystal growth and/or in a further stage influence polymorphic transitions or re-crystallization. The same authors recently published a good review concerning the influence of minor components, like DAG, on crystallization.

The first thermal effect during crystallization is the nucleation. Wähnelt et al. (1991) added 10% extra DAG to cocoa butter and observed a retarded crystallization with 1,2-DAG having more effect than 1,3-DAG. Other studies on cocoa butter equivalents have shown that DAG cause early crystallization, probably due to the nucleating species being DAG, further triggering the nucleation of saturated TAG (Cebula and Smith, 1992b). On the contrary, Loisel et al. (1998a) observed that distearoylglycerol did not influence the initial

crystallization. Gordon and Rahman (1991) concluded that 5% dilauroylglycerol extends the crystallization onset while dipalmitoylglycerol did not affect the nucleation.

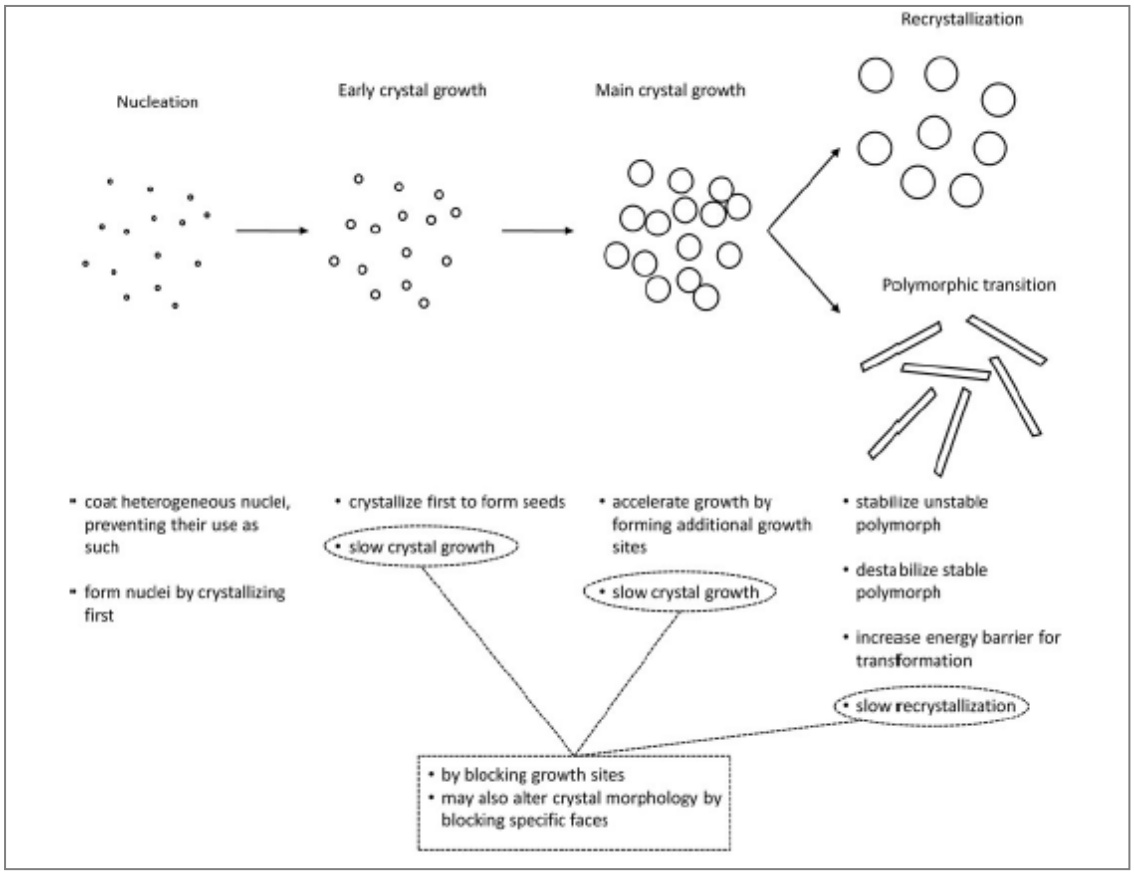


Figure 5.1 Modes of action of additives (after Smith et al. (2011))

The second stage of crystallization is the crystal growth. Smith et al. (1994) investigated the effect of lauric based molecules on trilaurin crystallization. Dilauroylglycerol isomers significantly reduce growth rate. Crystal morphology and the relative stability of the meta-stable phases increased. Growth rate was lower for samples containing the 1,3-isomer than for samples with the 1,2-dilauroylglycerol. Vanhoutte (2002) and Wright and Marangoni (2002) also observed a retarded crystallization and a decreased growth rate and the latter authors also showed that racemic purity is an important factor. Several authors like Gordon and Rahman (1991), Siew and Ng (1996), Smith and Povey (1997), Wright and Marangoni (2002) and Foubert et al. (2004a) concluded that the crystallization-enhancing or retarding role depends on the type of DAG as well as on the oil composition.

The more complex aspects of the crystallization properties of oils and fats arise from their polymorphism. By stabilizing a certain phase, transitions into another phase, often accompanied by product deterioration like fat bloom in chocolate and sandiness in spreads,

may be delayed or even prevented (Smith et al., 2011). Hernqvist and Anjou (1983) concluded that DAG can be useful as a β' stabilizer in margarine applications. This stabilizing effect is dependent on the chain length of the fatty acids as well as on the form (1,2-DAG vs 1,3-DAG). The effect is probably due to the formation of a fat crystal network containing DAG, rather than disturbing the crystal lattice. The longest stabilization occurred when the DAG chain length was similar to that of the fatty acids in the TAG. The stabilizing effect decreased with shorter chain length and longer chain length could create some co-crystallization problems.

Distearoylglycerol and also stearic acid retard the polymorphic transition after the second, major crystallization step in chocolate crystallization. DAG also retard polymorphic transitions in cocoa butter equivalents (CBE) on heating (Cebula and Smith, 1992a; Loisel et al., 1998b).

Reddy and Prabhakar (1986) have shown that DAG of sal fat either inhibited or delayed the phase transition of all the crystal forms of TAG, from their lower melting crystal forms to the next higher melting forms. The delay was more pronounced with the increase in DAG level from 5 to 15%.

Addition of 2 and 5% palm based DAG decreased the nucleation rate (insignificantly) and the crystallization rate and crystal growth mode of palm oil (significantly) (Saberi et al., 2011b).

Smith et al. (2011) state that besides an understanding of the processes of nucleation, crystal growth and polymorphic changes, the relevant mechanism is essential. In their review they list several possible mechanisms. By combining the information gained out of a multitude of studies, they derived some generic guidelines what to expect from minor components, indigenous or added:

- The effect of minor components is strongly dependent on the similarity between the bulk fat and the minor components: the greater the similarity, the greater the effect, especially in terms of acyl chain characteristics (length and number of double bonds). A similarity allows integration into the crystal matrix and/or addition onto a crystal growth site.
- An increasing degree of undercooling may reduce the effect of minor components. This is related to the different activation energies at different levels of undercooling.
- The concentration at which a minor component influences the physical properties of the bulk fat varies greatly with the mechanism involved.

5.2 Research strategy

Essential sensory and functional characteristics in fat based products are determined by different levels of structure within the crystal network, which are formed by their constituent lipid species. Knowledge of the isothermal phase behavior of cocoa butter is of utmost importance to optimize production processes and to maintain product quality (van Malssen et al., 1999). The isothermal crystallization of cocoa butter has been extensively studied as described in more detail in 1.6.2. Moreover, many researchers demonstrated that DAG (naturally present or intentionally added) affect the crystallization behaviour of TAG. Related to cocoa butter, only one study has been reporting on the effect of DAG on CB crystallization. Wähnelt et al. (1991) demonstrated that upon addition of pure isomeric DAG standards the α to β' phase transition delayed, the 1,2-DAG isomers being more reactive than the 1,3-DAG. Therefore, it seemed of interest to further explore the effect of CB based DAG added to a cocoa butter matrix.

The aim of this chapter was to study the impact of CB based DAG (up to 10%) on crystallization kinetics of cocoa butter, statically crystallized at 20°C. Another goal was to elucidate their effect on the crystallization mechanism of the CB TAG. Different experimental techniques (DSC, pNMR, real-time XRD, oscillatory rheology) were used to attain these goals.

As the macroscopic properties of fats and fat-containing products depend on their fat crystal network structure or microstructure, the microscopic properties were studied as function of DAG content.

5.3 Materials and methods

5.3.1 Samples

The CB (Barry Callebaut, Wieze, Belgium) defined in section 4.4.1 was blended with 1.25%, 2.5%, 5% and 10% CB DAG.

5.3.2 DSC

For the *isothermal crystallization behaviour* the following specific time-temperature program was applied: the sample was held at 80°C for 10 min to ensure a completely liquid state and erase the crystallization memory of the sample. Subsequently it was cooled at 10°C/min to 20°C and kept there for 240 min. Each analysis was executed in triplicate.

The principle of the s*top and return* DSC experiments is the interruption of the isothermal crystallization at specific interval times by heating the sample in order to generate melting profiles of the crystals present at the moment of interruption (Foubert et al., 2008). The procedure to study the isothermal crystallization behaviour was applied but the isothermal time was varied in different experiments prior to remelting. The area of the melting peak, which corresponds to the melting enthalpy, increased with increasing time at the isothermal temperature of 20°C, proportional to the degree of crystallinity, developed after a given time lapse. Accordingly, the degree of crystallinity as function of time at the crystallization temperature could be determined.

5.3.3 Solid fat content

For the crystallization kinetics experiments 3.5 ml of liquefied fat was transferred into NMR tubes and held at 70°C for 30 min to eliminate thermal history. These tubes are subsequently put in a thermostatic water bath at crystallization temperature. The amount of solid fat was measured at a preset time interval and a separate tube was used for each measurement. Each analysis was performed in triplicate.

5.3.4 X-ray diffraction analysis

As the wavelength of short X-rays is comparable to the size of atoms, they are ideally suited for probing the structural arrangement of atoms and molecules in a wide range of materials. If single crystals are available, X-ray diffraction analysis permits determination of the atomic positions in the unit cell with high precision, from which in turn the molecular conformation can be derived. Most of the information obtained by X-ray diffraction on crystals of TAG is, however, based on the study of polycrystalline materials using X-ray powder diffraction methods (Sato, 2004). Bragg's law describes the relationship between the incident angle θ (°) and the distance between the reflecting entities d_r (Å), depending on the wavelength λ (Å) used:

$$d_r = \frac{n'\lambda}{2\sin\theta}$$

in which n' is the order of diffraction. The physical background of this law is illustrated by Figure 5.2, representing X-rays inciding under small angles on fat crystals (Calliauw, 2008).

Depending on the detection angle relative to the incoming ray, wide angle X-ray diffraction (WAXD) and small angle X-ray diffraction (or scattering) (SAXS) can be differentiated, giving

rise to different spacings. WAXD is used to identify the lateral packing of fat crystals (e.g. α, β' and β) and is characterized by short spacings. SAXS is used to identify the longitudinal packing of fat crystals (2L and 3L) and is characterized by long spacings (Vereecken, 2010).

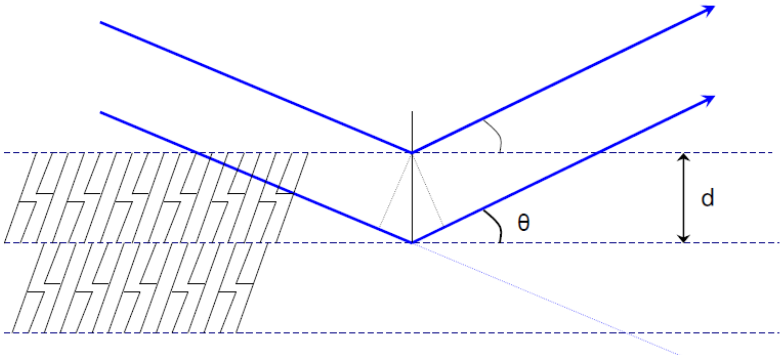


Figure 5.2 Principle of diffraction of X-ray beams in fat crystals (Calliauw, 2008)

For XRD analysis a D8 Advance diffractometer (Bruker, Germany) (λ Cu=1.54178 Å) equipped with a Vantec (Bruker, Germany) detector, and a TTK450 low-temperature Chamber & TCU 110 Temperature control Unit (Anton Paar, Graz, Austria) connected to a water bath (Julabo, Germany) was used. The measurement time was every two minutes between 1 and 13°2theta and also every two minutes between 15 and 27°2theta. Short and long spacings were determined using the Bragg law.
The XRD patterns were further analyzed using the PeakFit software (SeaSolve Software inc., Framingham, USA).

5.3.5 Determination of the microstructure by polarized light microscopy

The same procedure was applied as described in section 4.3.5.

5.3.6 Oscillatory rheology

Small deformation oscillatory experiments were performed on an AR 2000ex controlled stress rheometer (TA instruments, Brussels, Belgium) using the starch pasting cell as described by De Graef (2009). This SPC consists of a jacket, mounted on the instrument, a removable cup and an impeller. A gap of 5500 μm is used, which is larger than that of plate-plate or concentric cylinders geometries and reduces the risk of clogging. The melted sample (24 g) was transferred into the cup and subjected to a time-temperature profile. Samples were held at 70°C for 15 minutes to destroy all crystal memory and subsequently cooled to the isothermal temperature of 20°C at 10°C/min.

5.4 Results and discussion

The isothermal crystallization was performed at 20°C. From the time-temperature state diagram of Marangoni and Mcgauley (2003) it can be derived that at higher temperatures, CB crystallization is too slow and that at a lower degree of undercooling, the CB already starts to crystallize during the cooling to the isothermal temperature making it very difficult to evaluate the isothermal crystallization. At 20°C temperature, CB shows a typical two-step crystallization: in the first step, a part of the melt crystallizes into the α polymorph, while in the second step the α crystals transform into β' crystals via solid-solid transition (Dewettinck et al., 2004).

5.4.1 Isothermal crystallization behaviour as measured by pNMR

Figure 5.3 shows the isothermal crystallization curves of CB and the blends containing 1.25%, 2.5%, 5% and 10% DAG. All the samples clearly show a two-step crystallization process although some differences were observed between the samples. Figure 5.3a shows a detail of the solid fat increase as function of the first 50 minutes. It can be deduced that the higher the amount of DAG, the shorter the induction time and the higher the equilibrium solid fat of the first step. On the other hand, the length of the first plateau is inversely proportional to the amount of DAG. The re-crystallization from α to β', so the occurrence of the second step, was faster and the highest amount of solid fat was obtained at equilibrium. Adding 1.25% DAG already significantly changed the crystallization profile.

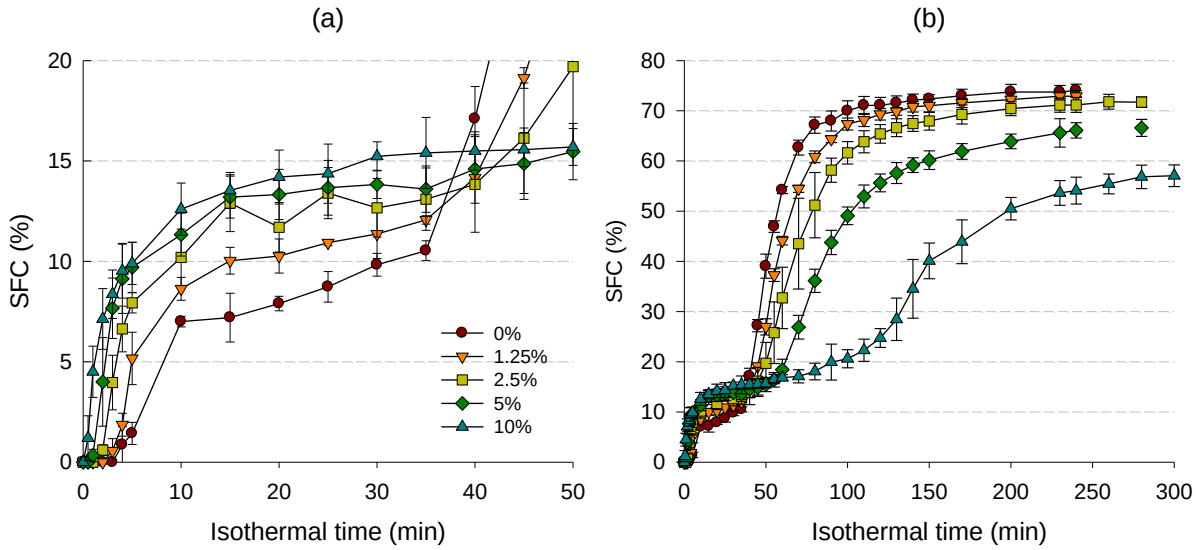
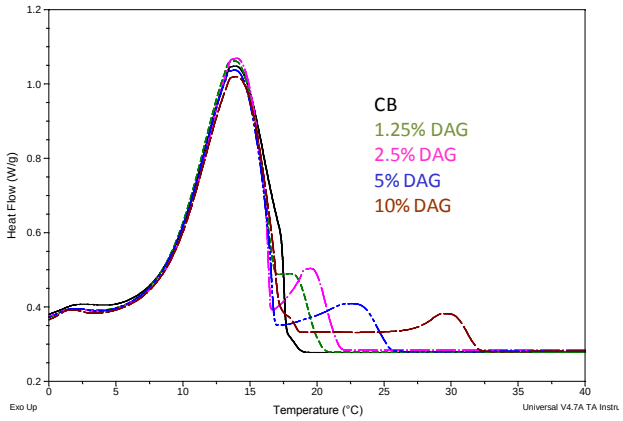


Figure 5.3 Isothermal crystallization curves at 20°C of CB and CB with 1.25, 2.5%, 5% and 10% CB DAG as measured by pNMR (a) detail of the first crystallization step (b) crystallization curve over the whole time range

5.4.2 Isothermal crystallization kinetics as measured by DSC

– *Crystallization onset and melting temperature (non isothermal)*

The crystallization onset temperatures of the different samples are listed in Figure 5.4. This temperature was determined by melting the samples at 80°C for 10 minutes followed by a cooling step at 5°C/min to -40°C. The onset was determined by the intersection of the baseline with the absolute highest tangent of the crystallization curve. The addition of 1.25% DAG already had a significant effect on the crystallization onset (Figure 5.4). The T_{onset} increased along with the appearance of a extra crystallization peak at higher temperatures. The onset of this secondary crystallization, extended to higher temperatures upon addition of more DAG. Campos et al. (2010) observed a similar increase upon addition of SSS to cocoa butter. They state that these higher melting TAG can form mixed crystals with those naturally present in CB. Therefore, the observations in this study are likely due to the high-melting DAG, the disaturated DAG forming mixed crystals with the high-melting CB TAG. The main crystallization peak remained unchanged when DAG were added.



% DAG	T_{onset} (°C)*
0	17.4±0.1[a]
1.25	20.3±0.2[b]
2.5	21.5±0.1[c]
5	25.1±0.4[d]
10	31.8±0.2[e]

*Letters indicate significant differences (α=0.05)

Figure 5.4 Onset crystallization temperature of CB and the CB samples with 1.25%, 2.5%, 5% and 10% DAG

After non isothermal cooling and subsequent heating at 5°C/min, the melting temperature (T_{onset} of the melting curve) was significantly lower for the sample containing 10% DAG (11.1°C) compared to the other samples (average values of 14.2°C) compared to the addition of DAG.

– *Isothermal crystallization at 20°C*

Figure 5.5 shows the isothermal crystallization curves obtained by DSC at 20°C. After cooling the temperature equilibrates to the crystallization temperature causing some sinusoidal variation in the crystallization curve. On top of this variation a more or less pronounced peak is visible. Because of its overlap with the temperature equilibration, it is

however impossible to integrate this peak (Dewettinck et al., 2004). This event is related to the crystallization of the α polymorph. After this peak, the curve returned to the baseline to increase again when the transformation to β' started. It can be seen that this increase was delayed when more DAG were added. This indicates that DAG may hinder the α to β' transformation. Additionally, the main crystallization peak shifted to longer crystallization times and was broader. At 1.25% and 2.5% DAG, a shoulder to the right side of the crystallization peak was observed. This may be indicative of a second lower melting fraction. The 5% and 10% curves show significant tailing meaning that more time was necessary for the β' phase to crystallize.

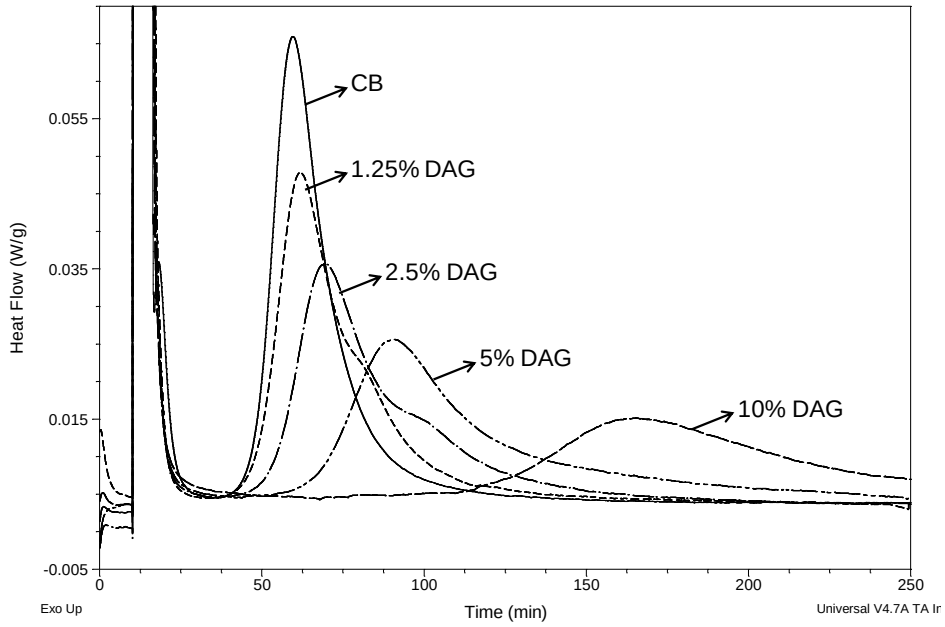


Figure 5.5 Isothermal crystallization curves at 20°C of CB and CB with 1.25%, 2.5%, 5% and 10% CB DAG as measured by DSC

– *Quantifying the crystallization kinetics*

The kinetics of the isothermal β' crystallization step can be described by the four parameters of the Foubert model. The start- and endpoint of the integration were determined with a calculation algorithm as described by Foubert (2003). By fitting the Foubert model, it is possible to determine a_f, t_{ind} and K. Parameter 'n' does not attribute to the mechanistic interpretation of the parameters, therefore the value of n is fixed at 6 to determine the changes in K (Sichien, 2007). The results from the Foubert fit are shown in Table 5.1.

Parameter a_f indicates the maximum amount of crystallization in the second crystallization step and is related to the amount of solid fat at equilibrium. The DAG imposed a negative, linear effect (R^2=0.996) on the amount of solid fat with increasing amount of DAG. So when more DAG are present, a lower percentage of TAG and/or DAG is able to crystallize under

the given conditions. However, as can be derived from Figure 5.3, it was expected that a lower amount of fat crystallized for 5 and 10% DAG within the given time frame. This may have influenced the Foubert fit as no equilibrium values were attained. Therefore, the interpretation of the Foubert parameter a_f should be done with care.

The t_{ind}, the time necessary to start the transformation from α-crystals to β'-form was slightly influenced upon addition of 2.5% DAG. At 5%, the t_{ind} almost doubled and when 10% was added the value even tripled compared to the value for CB.

Also the rate constant K was significantly affected indicating that DAG significantly slow down the β' formation.

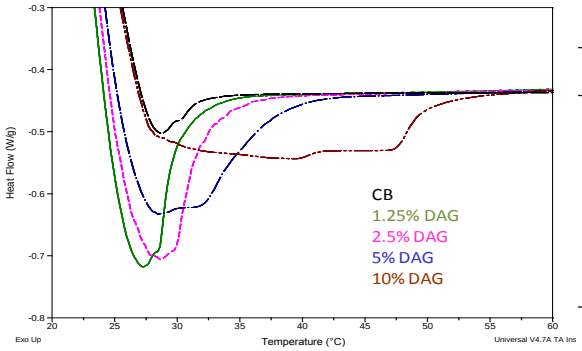
Table 5.1 The parameters from the Foubert model for CB, 1.25%, 2.5%, 5% and 10% DAG

	a_f (J/g)	t_{ind} (h)	K (h^{-1})
CB	**77.31±0.58** [a]	**0.47±0.01** [a]	**4.78±0.17** [a]
1.25 %	**71.43±1.13** [b]	**0.45±0.02** [a]	**3.52±0.30** [b]
2.5 %	**66.39±1.33** [c]	**0.49±0.01** [b]	**2.56±0.05** [c]
5 %	**58.29±1.71** [d]	**0.74±0.09** [c]	**2.04±0.17** [d]
10 %	**41.72±2.21** [e]	**1.57±0.36** [d]	**1.41±0.12** [e]

*Letters indicate significant differences (α=0.05)

- *Stop and return*

The principle of the stop and return technique is to interrupt the isothermal crystallization at different moments in time and subsequently heat the sample to obtain a melting profile of the crystals formed during the considered isothermal period. The melting profiles give an idea of the amount of crystallized cocoa butter (peak area) and the polymorph (peak maximum) in which the cocoa butter has crystallized (Dewettinck et al., 2004; Foubert et al., 2008). This method can provide some information about the beginning of the isothermal period. Therefore the melting curves after one minute isothermal crystallization at 20°C with the corresponding peak maximum and melting enthalpy are shown in Figure 5.6. In the case of CB, only a small fraction of cocoa butter crystals were formed after one minute at 20°C. The DAG clearly increased the nucleation as the melting enthalpy of the crystallized fraction increased. The melting peak is also becoming broader upon addition. For 5% and 10% DAG a melting range was defined. At 10% it was clear that a higher melting fraction, most likely the saturated DAG, was initiating the crystallization process.



% DAG	T (°C)	ΔH_{melt} (J/g)
CB	~28.5	0.74
1.25	27.3	3.90
2.5	28.6	5.13
5	28.2 – 31.9	5.12
10	28.2 – 47.2	6.55

Figure 5.6 Melting curves of the stop and return method for CB, 1.25%, 2.5%, 5% and 10% DAG after 1 minute isothermal crystallization at 20°C

Figure 5.7 presents some selected stop en return melting curves of CB and the CB enriched with 10% DAG. The results for the other samples are given in Annex IV-A.

The curves for CB showed a low-melting peak, with a peak maximum around 28°C up to 30 minutes. After 35 minutes of crystallization, this peak grew substantially but also shifted and broadened to a higher temperature. The peak maximum of the melting peak after 240 minutes crystallization at 20°C was around 32°C. This shift in temperature is again indicative for an α mediated polymorphic transition to β'. However, it is likely that some β' crystals are additionally formed directly from the melt (Foubert, 2003). Adding DAG to the cocoa butter, resulted in a slightly lower peak maximum (varying between 27.3°C to 28.1°C, for the different concentrations) of the lower melting fraction. It can also be seen that the substantial increase in peak area and the shift to higher temperatures was delayed by the DAG. The peak maxima after 240 minutes crystallization were also substantially lower compared to the CB.

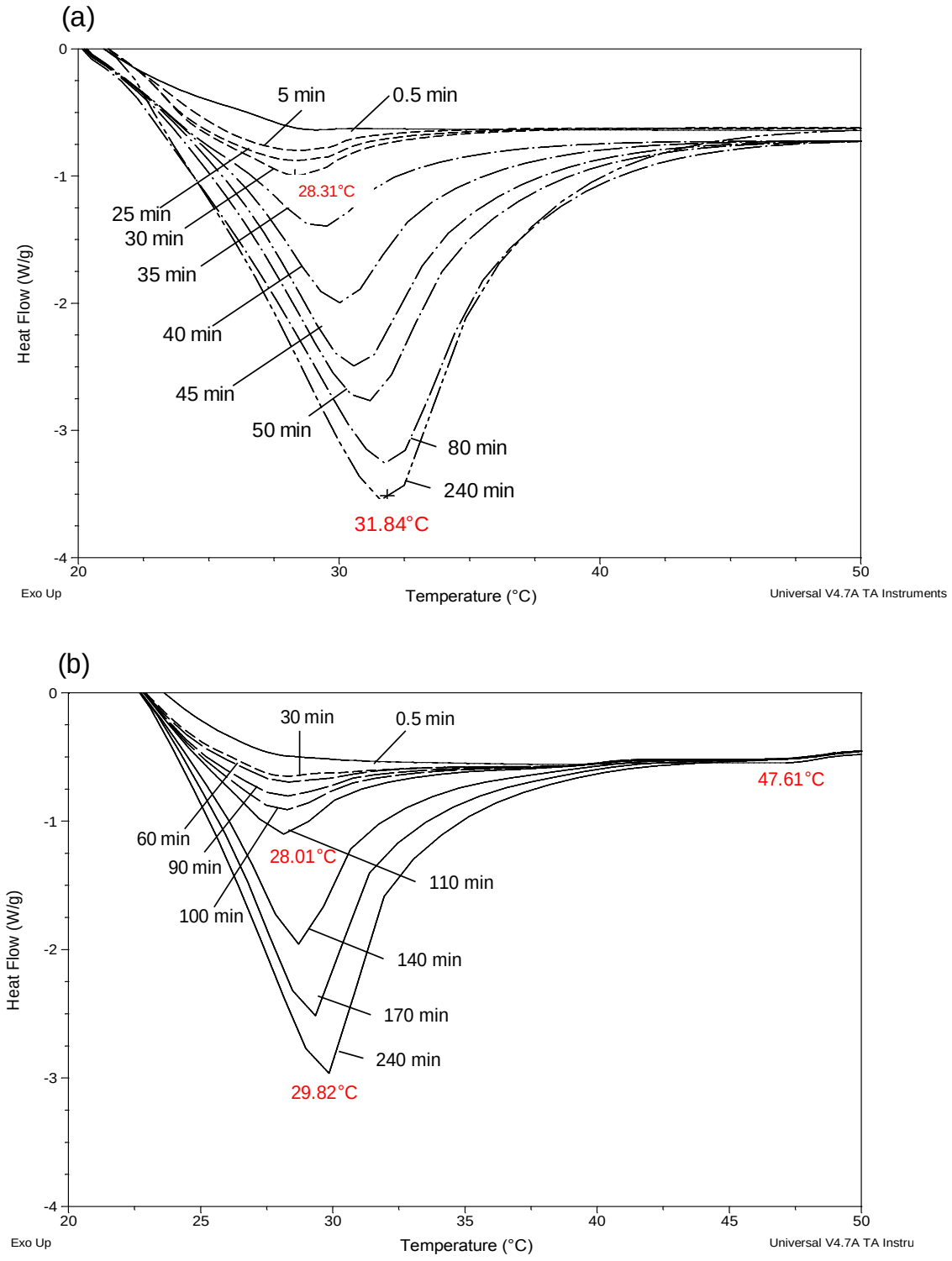


Figure 5.7 Overlay of stop and return melting curves of (a) CB and (b) 10% DAG at 20°C Isothermal crystallization times and some peak maxima are indicated

The different melting curves were integrated and the results were plotted as function of time as shown in Figure 5.8. The curves were very similar to the results obtained by pNMR in Figure 5.3. More crystals were formed during the first crystallization step when more DAG were present but on the other hand, the transition to the second step was significantly

delayed: e.g. for CB this event occurred between 30 – 35 min, between 35 – 40 min for 1.25%, ~45 min for 2.5%, ~60 min for 5% and ~110 min for 10%. Also, the melting enthalpy of the second fraction decreases with increasing amount of DAG coinciding with the lower amount of solid fat in the isothermal crystallization curves as determined by pNMR.

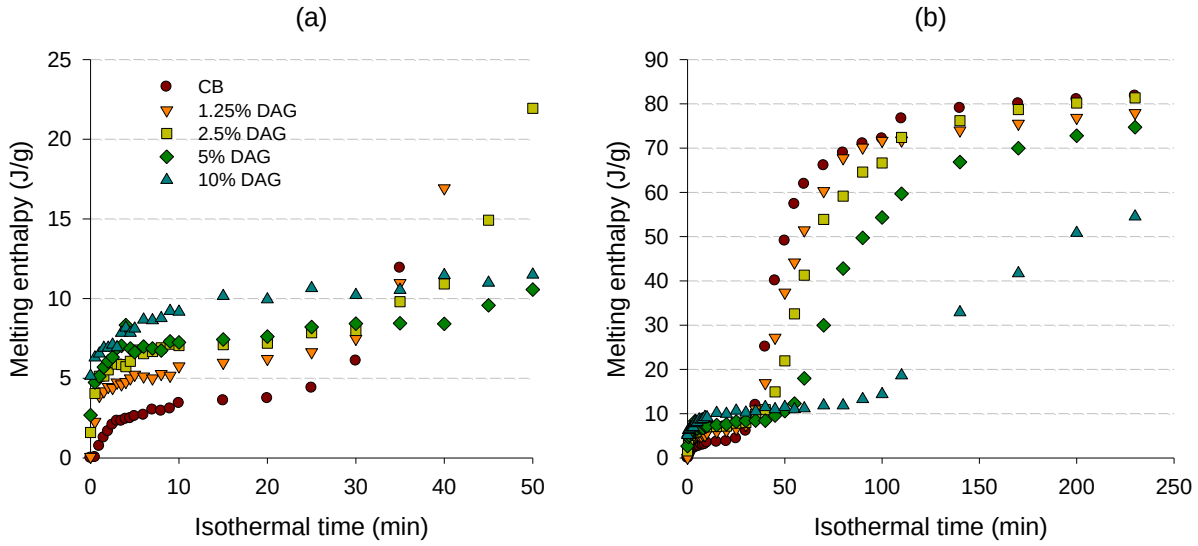


Figure 5.8 Melting enthalpy as function of isothermal crystallization time at 20°C for CB, 1.25%, 2.5%, 5% and 10% DAG

5.4.3 Isothermal crystallization behaviour as measured by oscillatory rheology

As stated by De Graef (2009), pNMR and DSC provide information on primary crystallization only while oscillatory rheology provides information on all three steps of the crystallization process, namely primary crystallization, microstructural development of the fat crystal network and macroscopic properties.

To monitor crystallization by rheograms, two parameters are of major importance. The first is the complex modulus |G*|, defined as the ratio of the shear stress to the applied deformation and consists of a real and an imaginary component, respectively, G' (elastic, storage or in-phase modulus) as a measurement of the solid nature and G" (the viscous, loss or out-phase modulus) as an indication for the fluid character of the viscoelastic system. The phase angle δ encompasses both G' and G", evaluating in a quite sensitive way the viscoelastic changes of complex systems. In a pure viscous system, such as completely melted oil, δ equals 90°. Once nucleation occurs and crystals start to grow, δ starts to decrease as the solid character of the crystallizing oil increases. This decrease can be quite drastically. At a δ above 45°, the sample displays a predominantly fluid character, while for values lower than 45°, the solid character prevails. Eventually, when the system becomes fully crystallized, δ approaches zero (Toro-Vazquez et al., 2004).

Toro-Vazquez et al. (2004) have used oscillatory rheology to study the crystallization behaviour of CB under static and stirring conditions and they attributed the evolution of phase angle to the crystallization in different polymorphic forms. De Graef (2009) did the same for the crystallization of palm oil.

The complex modulus and phase angle as function of isothermal time for the different DAG concentrations are shown in Figure 5.9. The point at which the complex modulus starts to increase can be considered as the onset of crystallization. This occured earlier with increasing amounts of DAG. For 10% DAG, the sample started to crystallize during cooling as the complex modulus already showed a higher value at the beginning of the isothermal period. Again, a two-step crystallization process can be distinguished: after the crystallization onset the complex modulus showed a fast increase, slowing down to the end of the first crystallization step. This was then followed by an increase with a steeper slope to level off at the end of the 2nd step. The increase in complex modulus is not only due to the primary crystallization, but also due to the microstructural development by aggregation (De Graef, 2009). Figure 5.9 clearly shows higher complex modulus values in the first crystallization step for higher amounts of DAG indicating that during α crystallization, a stronger network was formed when more DAG were present. The occurrence of the second step, related to the α to β' transformation, was inversely related to the amount of DAG and so CB was the first to reach a plateau value. Up to 2.5% DAG, the differences are rather limited. At 5 and 10%, the first crystallization step took considerable more time and at 5% a stiffer network will be formed compared to 10% DAG.

The changes in the complex moduli coincide with the timescale of the variations in phase angle. The sample with 10% showed a phase angle of 75° at the start of the isothermal period indicating that crystallization already occurred during the cooling. For all samples, although over different timescale, the phase angle decreased drastically to values between 20 and 30° indicating that during α crystallization most of the liquid material did already crystallize. This decrease slowed down but at the second crystallization step, a steep decrease to a final value of around 1° was observed. For CB and 1.25% DAG this occurred in a time frame of ~60 minutes, while the samples with 2.5%, 5% and 10% DAG needed around ~70, ~80 and ~120 minutes respectively. Important to notice is that the curves of 2.5% and 10% showed an increase which can be related to the amount of heat released during the crystallization process. The crystalization heat can not be removed immediately, causing temporary temperature rise. Crystals melt locally, loosening the network structure and thus leading to an increased phase angle (De Graef, 2009; Toro-Vazquez et al., 2004).

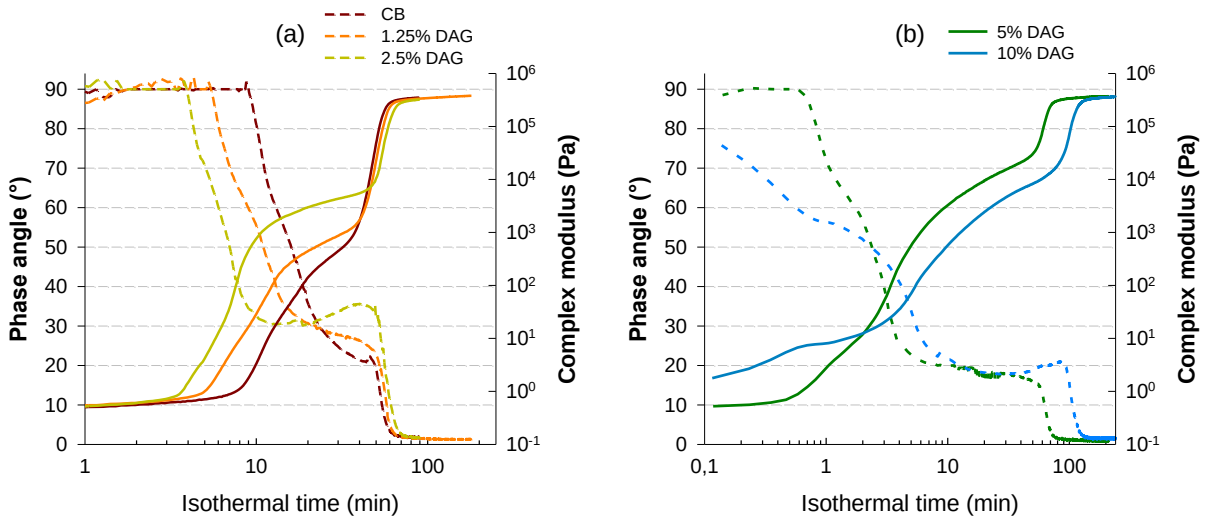


Figure 5.9 Evolution of the phase angle δ (---) and complex modulus |G*| (—) as function of isothermal crystallization time for (a) CB, 1.25% and 2.5% DAG and (b) 5% and 10% DAG

5.4.4 Isothermal crystallization behaviour as measured by XRD

By real time XRD, the short and long spacings as function of time are determined providing unambiguous phase information at each moment in time (Dewettinck et al., 2004). Wide angle reflections were used to determine the characteristic short spacings, which provide information regarding the lateral packing of fatty acid chains within the lamella or the layer thickness (d). Small angle reflections are used to identify the longitudinal packing of fat crystals (2L and 3L) and are characterized by long spacings (Campos et al., 2010; Fredrick et al., 2008).

The development of the crystalline structure of the samples at 20°C, simultaneously recorded by WAXD and SAXS, as function of time in time is shown in Figure 5.10, Figure 5.11 and Figure 5.12. In Annex IV-B, the WAXD patterns are presented in 2θ values as function of the isothermal time. Literature data (Campos et al., 2010; Dewettinck et al., 2004; Loisel et al., 1998a; van Malssen et al., 1999) were used to allocate the different peaks to the different polymorphs.

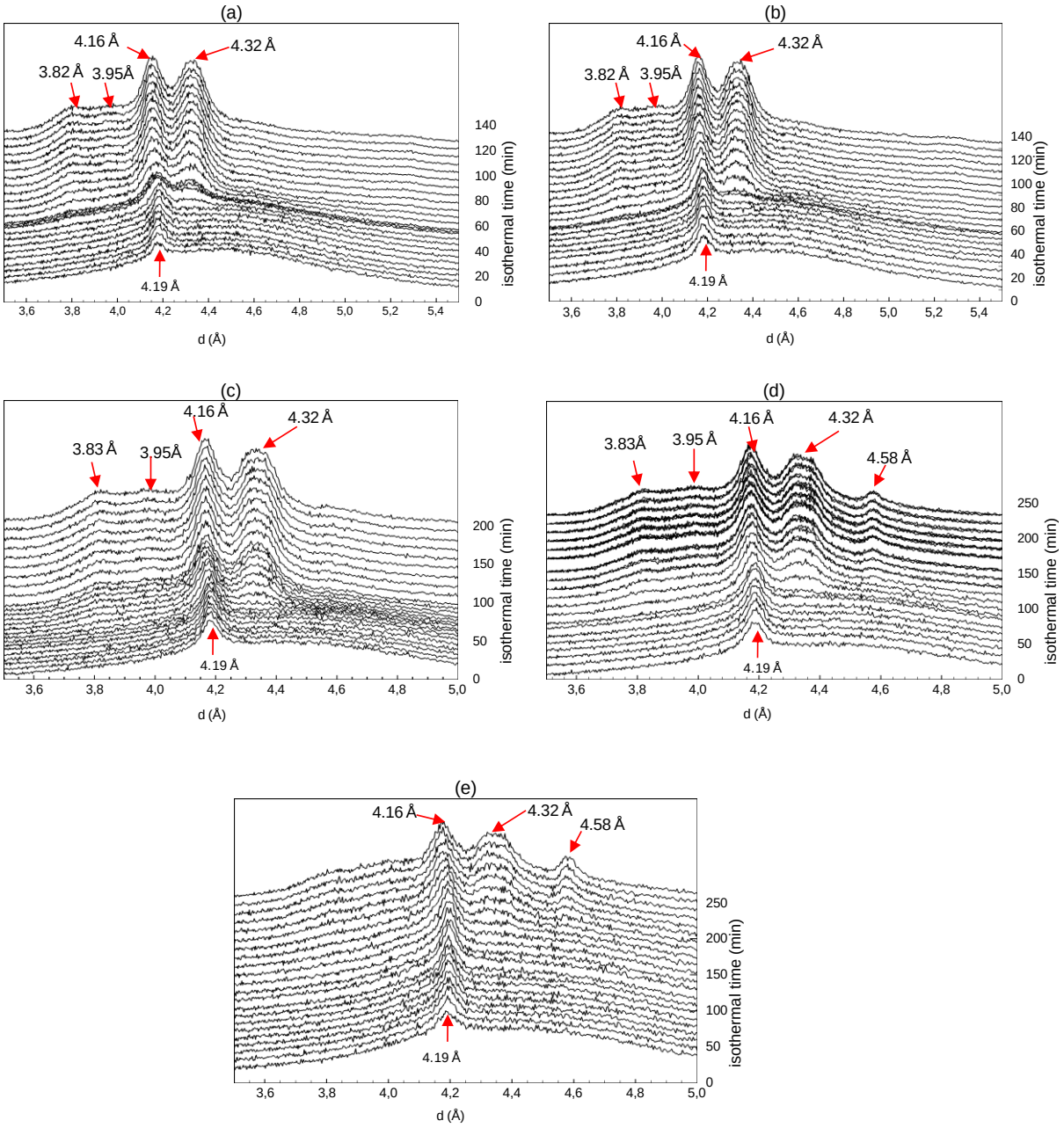


Figure 5.10 WAXD patterns of (a) CB, (b) 1.25%, (c) 2.5%, (d) 5% and (e) 10% DAG isothermally crystallized at 20°C

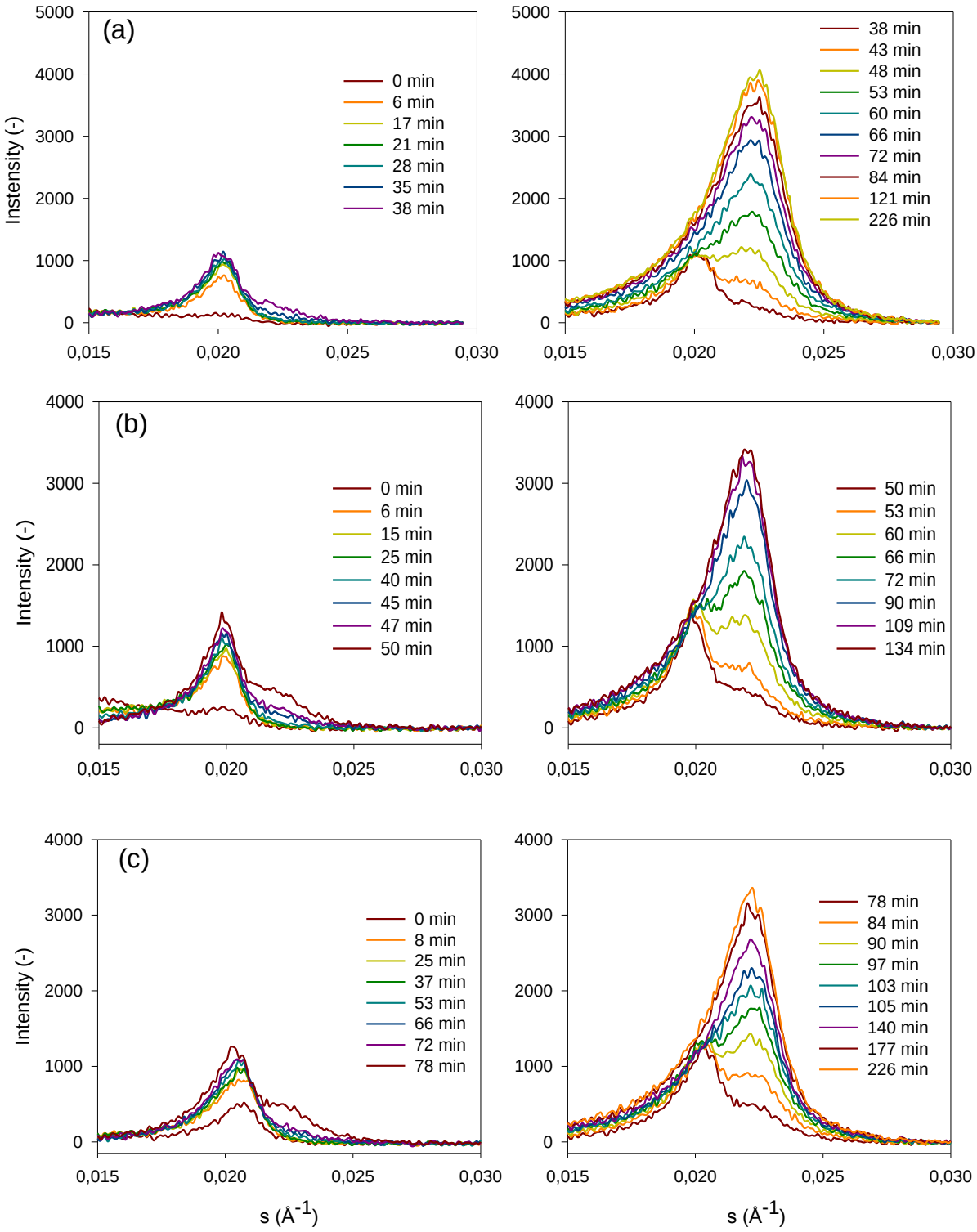


Figure 5.11 SAXS diffraction patterns of the isothermal crystallization at 20°C for (a) CB; (b) 1.25% and (c) 2.5% DAG as function of time

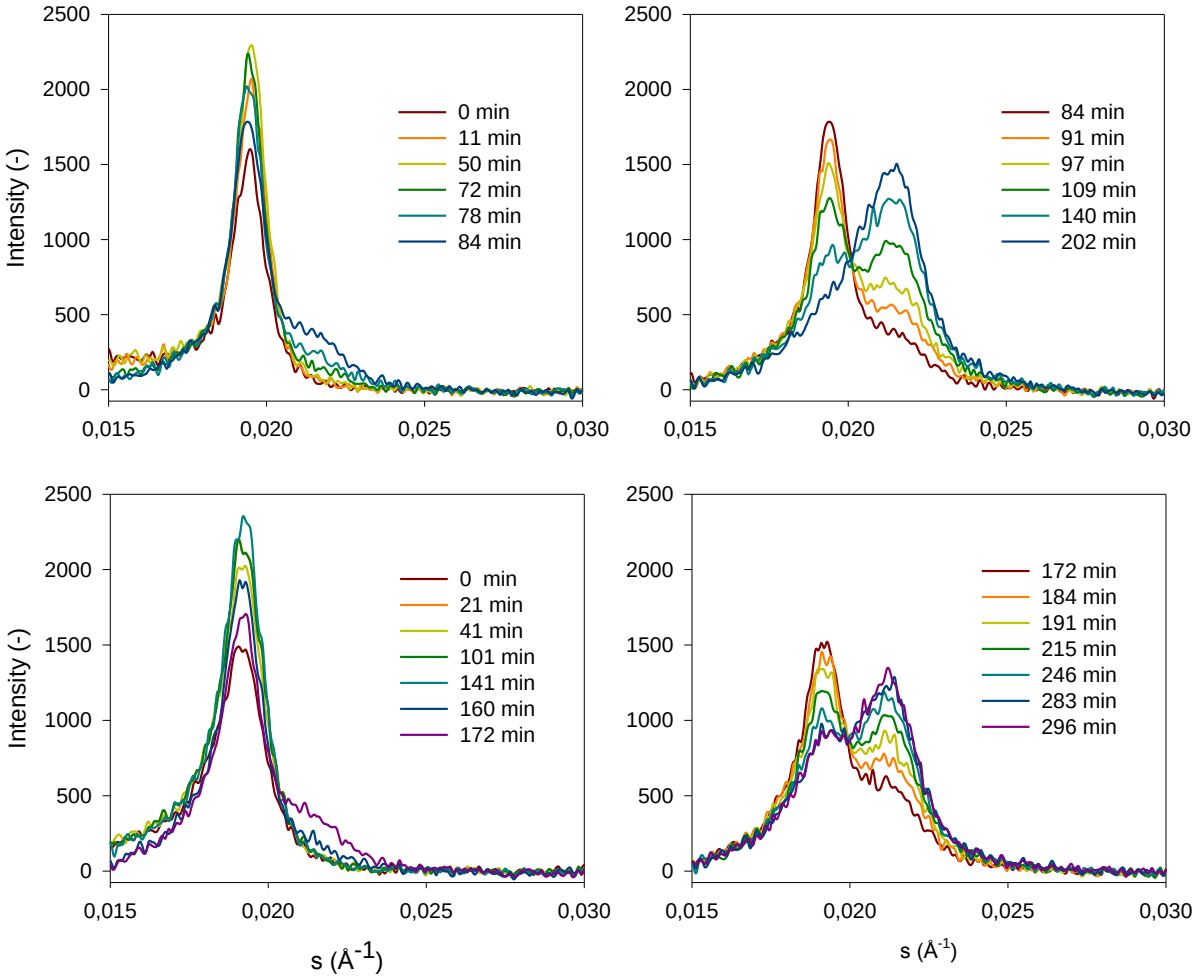


Figure 5.12 SAXS diffraction patterns of the isothermal crystallization at 20°C for (a) 5% and (b) 10% DAG as function of time

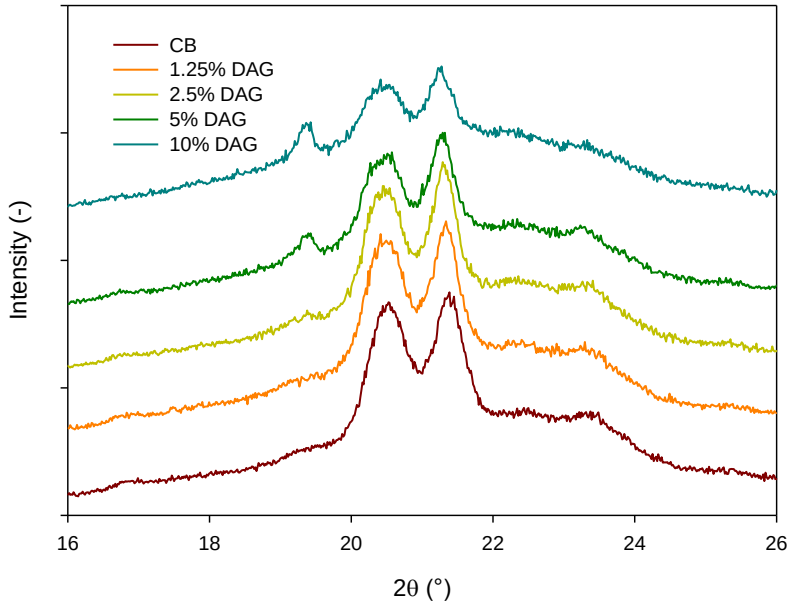


Figure 5.13 XRD diffraction patterns of CB; 1.25%; 2.5%; 5% and 10% at the end of the isothermal period

For all the blends, a single peak with a short spacing of 4.19 Å and a long spacing between s= 0.0201 $Å^{-1}$ (d= 49.8 Å) for CB and 0.0191 $Å^{-1}$ (d= 52.2 Å) for 10% DAG were observed. This is characteristic for the formation of a hexagonal chain packing, the most unstable packing of the acylglycerol chains which is unequivocally connected to an α polymorph. The α polymorph has a double chain length structure conformation with no tilt (Campos et al., 2010). Up to 2.5%, no α crystals were formed during cooling as the first frame was showing a liquid signal (Figure 5.11, figures on the left) while for 5% and 10% α crystals are already present when isothermal temperature was reached (Figure 5.12, figures on the left). It can be seen from the SAXS patterns that after a certain time, depending on the amount of DAG, this peak started to vanish, coinciding with the appearance of a second peak around s=0.0222 $Å^{-1}$ (d= 45.2 Å) for CB and 0.0213 $Å^{-1}$ (d= 47.0 Å) for 10% DAG. Concomitantly, in the WAXD diffraction patterns the peak at 4.19 Å shifted to 4.16 Å and a second major peak at 4.32 Å appeared. This can be assigned to a β' transformation with an inclined (with respect to the lamellar interface) double chain length structure (Campos et al., 2010). This was observed in all blends, however the time of appearance was different. For CB the shoulder of the β' peak in the SAXS patterns was observed after 38 min and this increased proportionally with the amount of DAG to approximately 50, 60, 84 and 172 min for 1.25, 2.5, 5 and 10% DAG respectively. The β' peak grew over time indicating an increase of the β' polymorph.

Up to the addition of 5% DAG, extra peaks were observed in the WAXD patterns at 3.82 Å and 3.95 Å. As illustrated by Van Malssen et al. (1999) the β' form rather exists as a phase range as different β' XRD patterns were observed. A characteristic WAXD reflection pattern for the different samples at the end of the isothermal period is shown in Figure 5.13. The observed patterns up to 2.5% DAG were close to the patterns reported for β_{IV}. Remarkable was the appearance of an extra peak around 4.58 Å after a certain time during the β' phase. This peak is characteristic for the presence of the β form (Marangoni and McGauley, 2003). The appearance of the peak at 4.58 Å was also observed after 2 days storage of CB at 20°C in the study of Campos et al. (2010). They concluded that, as CB reaches an equilibrium SFC after 120 min, the formation and growth of the newly observed β phase is at the expense of the existing β' through solid state polymorphic transformations. However, in this study, it was clear that the SFC or melting enthalpy still increased as function of measuring time. But as stated by Van Malssen et al. (1999) the β phase is formed via the phase transformation from β', so this is the most likely mechanism.

An interesting observation in the SAXS patterns (Figure 5.11) is that the curves for 5% and 10% DAG pass through one single point, known as an isosbestic point. An isosbestic point

indicates that the total crystalline volume fraction does not change during the α to β' transformation (Dewettinck et al., 2004). Or, every β' crystal is resulting from an α, so no β' crystals are formed from the melt, as long as the isosbestic point exists (Calliauw et al., 2008). An isosbestic point has been observed for palm oil crystallization at 25°C (Fredrick et al., 2008), milk fat (Vanhoutte, 2002) and cocoa butter (Dewettinck et al., 2004). However, Calliauw et al. (2008) didn't observe this phenomenon in their study comparing a mildly and strongly refined cocoa butter.

The SAXS patterns (Figure 5.11) show that up to 2.5% addition of DAG, α is transformed to β' but no isosbestic point could be observed. At the beginning of the isothermal period, the α peak increased to a rather constant intensity. After a certain isothermal time, a small shoulder appeared, related to the β' crystallization. Quite rapidly, the intensity of the β' peak surpassed the α peak, to resolve it completely after e.g. 47 min for CB. The addition of the DAG increased this time, indicative of a slower transformation to β'. The intensity of the β' peak further increased as β' will directly crystallize from the melt.

For the addition of 5 and 10% the beginning of the crystallization process is similar with the formation of the metastable α crystals but as an isosbestic point is observed the β' only grow at the expense of the α crystals and no crystals are formed directly from the melt.

Although strictly, the intensity of the diffraction does not permit an accurate quantification of the amount of crystallization (Calliauw et al., 2008), it should be noted that the intensity of the α-diffraction of the samples up to 2.5% is much lower compared to the ones with higher amounts for DAG, indicating a higher amount of crystallization during this first crystallization step.

– *The crystal structure*

When an oil sample is cooled below its melting point, a phase change will occur as the TAG which are in random thermal motion in the liquid will orient and align with each other in characteristic lateral packing and longitudinal stacking forming lamellae. Series of lamellae form domains, which in turn stack to form single crystallites. These crystals aggregate to form clusters, further interacting with each other to create flocs to ultimately form three-dimensional networks (Campos et al., 2010). This is visualized in Figure 5.14. Small angle reflections provide information about the crystal structure. The simplification of the Scherrer equation taking into account XRD at very small angles allows an approximation of the thickness of the crystals formed (Lopez et al., 2002). The center of the peak was used to calculate the lamella longitudinal size while the full width at half maximum can be related to the structural organization of TAG molecules into domains.

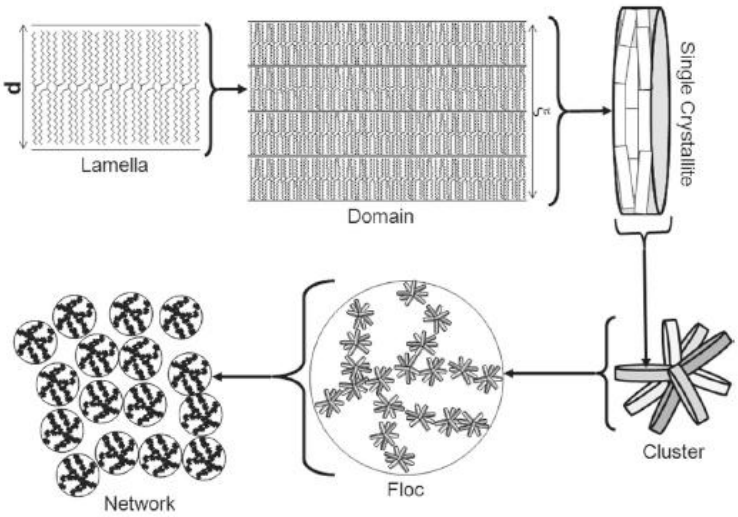


Figure 5.14 Schematic representation of the different levels of structure in crystallized fats (Campos, 2010)

To determine the center and the full width at half maximum a Lorentzian curve (Campos et al., 2010) using PeakFit Software was fitted to the small angle reflections. As function of time, the diffraction patterns evolved from one peak for the α form to more complicate patterns when transformation to the β' form occurred. From the moment a shoulder was detected in the patterns an attempt was made to integrate the peak with the software. The fit was evaluated by determining the residual sum of squares and the R^2. The results of the analyses are shown in Figure 5.15. The long spacing of CB was around 49.8 Å when crystallized at 20°C. The lamella size of 1.25% DAG was slightly higher with values around 50.4 Å and that of 2.5% was lower with values around 49.2 Å. Adding 5% or 10% DAG resulted in significantly larger long spacings, with values up to 51.3 Å and 52.2 Å. Campos et al. (2010) observed similar values upon addition of tristearin to CB. The domain size, derived from the full width at half maximum, was quite similar for the different samples with values around 600 Å, however the samples with 5% DAG showed higher values around 950 Å. This broader width may indicate that the formed crystals are smaller as the peak width is inversely related with crystallite size (Lopez et al., 2002).

As the crystallization process progressed, crystal structures changed, which was reflected in a decrease in lamella and domain size. In the diffraction patterns, the transition to the β' forms was notified by the appearance of a diffraction peak with smaller long spacings. This was observed for all samples however, differences were observed in time of appearance. At the beginning of this transformation, the peaks were often difficult to resolve, impeding the peak integration, therefore data were somewhat scattered (Figure 5.15b and d). When the β' peak became predominant values reached an equilibrium value. Both lamella and domain

size decreased as the result of an inclined, double chain length structure, characteristic for β'. They both showed an increasing trend as function of DAG concentration. The lamella and domain size of CB at equilibrium were 45.2 Å and 350 Å respectively while the CB with 10% DAG showed values of 47 Å and 550 Å, keeping in mind that transformation was still in progress.

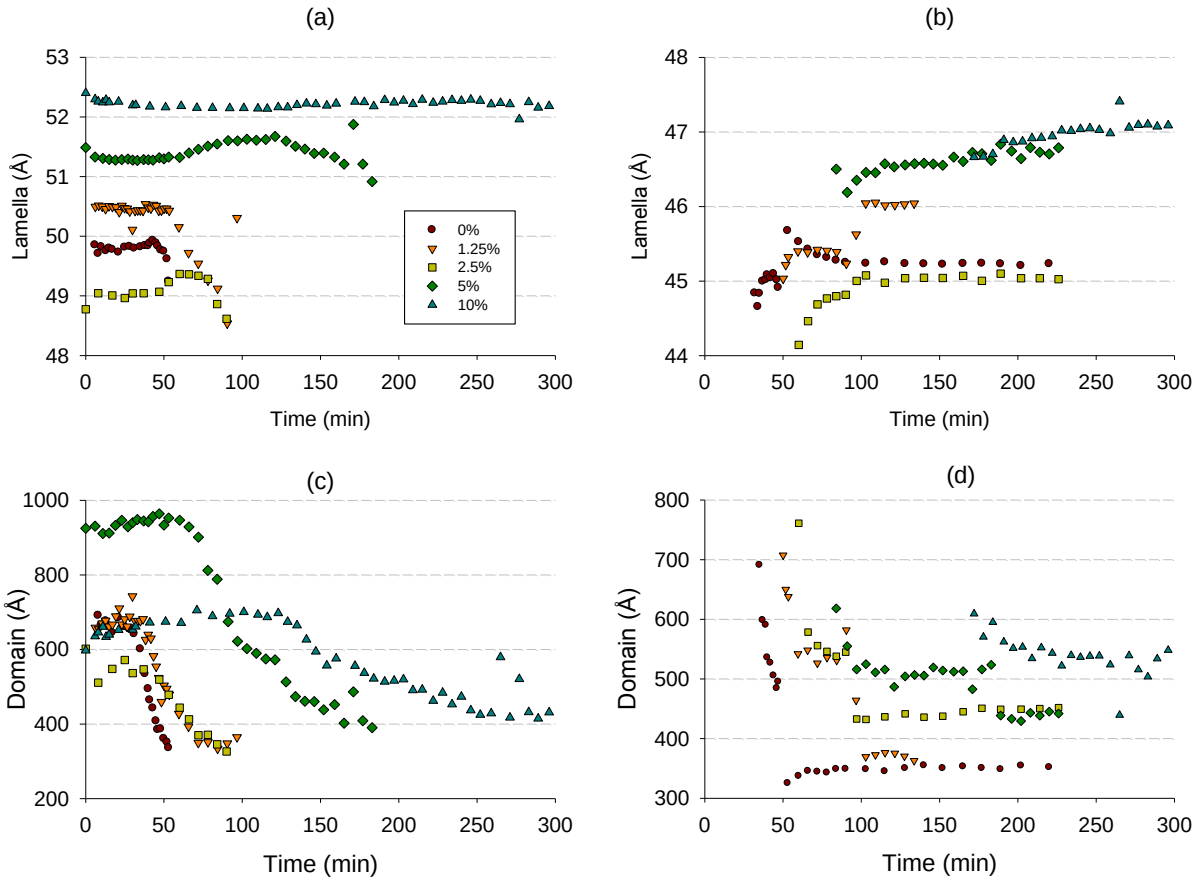


Figure 5.15 Lamella and domain size for α (a-c) and β' (b-d) form respectively derived from the long spacings during isothermal crystallization of CB, 1.25%, 2.5%, 5% and 10% DAG

5.4.5 Influence on microstructure

PLM allows the study of the microcrystalline structure, yet at a much larger length-scale relative to XRD, enabling the vizualisation of the aggregation of domains in single crystallites and their further aggregation into clusters (Campos et al., 2010).

In the previous chapter, section 4.4.3, PLM images revealed significant different microstructures when major amounts of DAG were added to CB. Moreover, as microstructure is a direct consequence of the crystallization kinetics, it was visualized by PLM. The different blends from this study were therefore monitored at regular base during a

storage period of 5 weeks. An overview of some representative pictures after 1 day, 1 week, 3 and 5 weeks is given in Figure 5.16. The procedure is described in section 5.3.5.

For CB, the first crystals were observed ±2 minutes after attaining 20°C. The addition of CB DAG decreased the induction time. First, nuclei were already observed during the cooling phase for 5% DAG (around 27°C) and 10% DAG (around 40°C). The crystals had a granular appearance, characteristic for the α polymorph (Marangoni and McGauley, 2003). The crystallites grew and after 24 hours at 20°C, a dense network was observed for all samples, making it difficult to distinguish crystallites. Marangoni and McGauley (2003) and Campos et al. (2010) observed clustering during the first 24 hours at 20°C and assigned this as an evidence of a polymorphic transformation from α to β'. In this study, even after four days storage, the continuous phase with the same morphology was still observed (results not shown). Probably, the network was too dense to visualize the clustering. After 7 days, the continuous phase still had the same morphology, but spherulites and larger microstructures were observed. Around seven days, clusters of spherulites grow into each other, resulting in larger microstructures (Brunello et al., 2003). This coincides with the β' to β polymorphic transition (Marangoni and McGauley, 2003). The larger microstructures showed a granular center with leaf-, feather-like structures at the periphery. The size of the granular center seemed to increase with an increasing amount of DAG.

After 3 weeks storage, the larger microstructures became predominant with sizes between 600 – 1000 μm. The CB still demonstrated a more leaf-like periphery while the more feather-like type of crystals were observed in the other samples. The granular center of the CB with 10% DAG was still larger compared to the lower concentrations DAG. The larger microstructures also started to grow into each other.

For CB, the polymorphic transition from β' to β is complete after 35 days and the crystals are in the β form (Brunello et al., 2003). The extremely large microstructures, with mainly a feather-like appearance, were very similar for CB and up to 5% DAG. For 10% DAG, large microstructures remained more circular, more granular material was observed and feather-like structures were considerably smaller.

It can be concluded that when samples were subjected to the same time-temperature treatment, differences in microstructure were relatively small up to an addition of 2.5% DAG. The sample with 5% DAG showed some subtle differences while for 10% DAG more circular, less feather-like microstructures were observed after a storage period of 5 weeks.

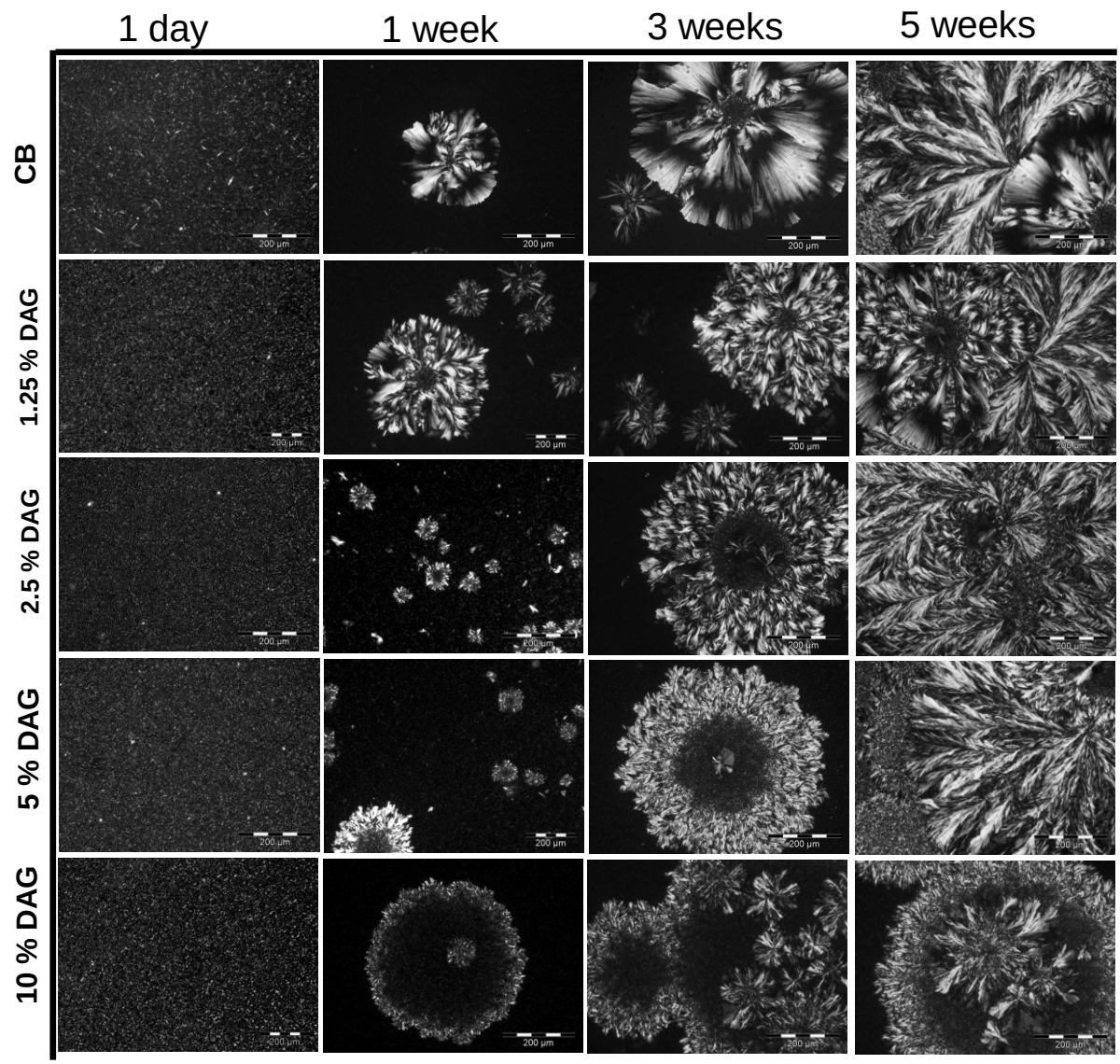


Figure 5.16 Polarized light micrographs of CB, 1.25% DAG, 2.5% DAG, 5% DAG and 10% DAG crystallized isothermally at 20°C. The microstructure was imaged after 1day, 1 week, 3 weeks and 5 weeks

5.4.6 Discussion

In this paragraph, the information obtained by the different techniques is summarized and listed to describe the effect of DAG on the isothermal crystallization of CB at 20°C.

One should keep in mind that comparison of different equipment to monitor crystallization should be done with caution. Differences in sample weight or volume, equipment design and its impact on thermodynamics of the system and heat and mass transfer conditions existing in each measurement device may affect crystallization to a different extent (Toro-Vazquez et al., 2004). For example, the differences in crystallinity obtained by different measuring

techniques (pNMR vs. DSC stop and return) can be attributed to the fact that the different techniques measure different physical features of the material (Dewettinck et al., 2004).
The different techniques revealed a two-step crystallization process up to an addition of 10% DAG. The process was split up in different stages and for each step, the most important observations were listed.

- ***Stage I: Supercooled melt ($t_I \leq$ 0 min)***

≤ 1.25% DAG: no crystallization during cooling

  - No crystalline diffraction patterns
  - No crystals in PLM

≥2.5%: crystallization during cooling

  - Diffraction pattern in SAXS
  - Phase angle <90° for 10%
  - Visual observation of crystals in PLM (not for 2.5%)

- ***Stage II: Direct crystallization of α ($0<t_{II} < t_{\beta'}$)***

For all samples:

  - WAXD diffraction at 4.19 Å: α
  - SAXS diffraction: 49-50 Å up to 2.5%; 51-52 Å for 5 and 10%: 2L
  - Increase of the amount of solid fat to plateau value in pNMR
  - Increase of the melting enthalpy of the melting curves in stop and return to a plateau value
  - Drastic decrease of phase angle in oscillatory rheology
  - Network formation as complex modulus increased

t_{II} was proportional to the amount of DAG: prolonged α lifetime as function of DAG
Amount of solid fat and melting enthalpy were directly proportional and the phase angle inversely proportional to the amount of DAG: more α crystallization as function of DAG

- ***Stage III: α to β' polymorphic transition ($t_{III} = t_{\beta'}$)***

For all samples:

  - WAXD diffraction pattern: ~3.82 Å, 3.95 Å, 4.16 Å, 4.32 Å: β'
  - SAXS diffraction pattern: appearance 45 – 46Å (up to 2.5%); ~47Å (5% and 10%): 2L
  - Significant increase solid fat content in pNMR
  - Significant increase melting enthalpy in stop and return

- Melting peak in stop and return shifted to higher temperatures, most clear for CB, less pronounced when DAG were present
- Oscillatory rheology: significant decrease phase angle, significant increase complex modulus
- $t_{III} = t_{ind}$ Foubert model: longer induction time as function of the amount of DAG

- ***Stage IV: Growth of β' ($t_{\beta'} < t_{IV} < 240$ min)***

For all samples:

- Foubert parameters: the rate of β' formation (K), maximum amount of crystallization (a_f) decreased as function of DAG
- Oscillatory rheology: maximum network strength and all the liquid material did solidify

≤ 2.5% DAG:

- WAXD and SAXS: β' 2L
- No isosbestic point in SAXS patterns: α → β' + melt → β'
- Solid fat content in pNMR leveled off to ~ 70% solid fat
- Melting enthalpy in stop and return leveled off to 75 J/g

5% and 10% DAG: crystallization during cooling

- WAXD: extra diffraction peak at 4.58 Å → characteristic for β, β' structure remained predominant
- SAXS: isosbestic point : β' crystals grew at the expense of α, no direct formation form the melt
- Solid fat content still increasing within the time frame
- Melting enthalpy still increasing within the time frame

The fact that CB shows a two-step crystallization process and first crystallizes in the less stable α polymorph can be explained as follows. The α polymorph has a lower critical activation free energy for nucleation and thus a higher nucleation rate despite the larger difference in chemical potential and thus the larger driving force for crystallization for the more stable polymorphs (Dewettinck et al., 2004). Therefore, the α polymorph is kinetically favoured to crystallize first. When DAG were added to CB, the induction time decreased and more α crystals were formed. The blend with 5 and 10% DAG even crystallized during cooling. This can be explained by the fact that the CB DAG contain a high-melting fraction, the disaturated DAG. The latter change the saturation conditions of the melt. They become rapidly undercooled, they nucleate and grow and act as crystal seeds to CB TAG crystallization. They form effective catalytic impurities for heterogeneous nucleation. The

larger amount of nucleation sites implies the simultaneous formation of a larger quantity of smaller crystals and therefore a larger amount of TAG will crystallize in the α polymorph. After some time the rate of α crystallization decreases drastically owing to the lowering of the supersaturation.

Similar to the introduction of tristearin (SSS) to cocoa butter in the study of Campos et al. (2010), the inclusion of the predominantly high-melting DAG into the crystal lattice of nuclei may introduce a certain degree of disorganization during the stacking of lamella in the very early stage of crystallization. The synthesized CB DAG contained a mixture of 1,3- and 1,2/2,3-DAG isomers. The directionality of the FA chains in the crystalline state differs between DAG isomers. In 1,3-DAG, the fatty acid chains extend in opposite directions and the molecules tend to pack in monolayers. Conversely, in the 1,2(2,3)-DAG isomer, chains extend in the same direction, resembling the tuning fork arrangement characteristic for TAG conformation (Wright and Marangoni, 2002). Moreover, it has been confirmed that the two isomeric forms have different crystal structure and polymorphism. The 1,2-DAG have an α polymorph that can further transform to the β' form. The 1,3-DAG only crystallize into β forms with triclinic packing (Craven and Lencki, 2011; Shannon et al., 1992). Consequently, it can be assumed that different configurations will be present. During the nucleation step, it will be mainly the SS type of DAG that infringes the crystal structure. The presence of saturated fatty acids resulted in molecules that were spatially bigger compared to molecules where a monounsaturated fatty acid is present. In this study it was seen that when 5 or 10% DAG were present, this resulted in differences in crystal lamella and domain size.

Despite the lower induction times for α crystallization, DAG prolonged the α-lifetime and delayed the polymorphic transition to β'. The molecular structures of DAG, including fatty acid chain length and the positions of the fatty acids on the glycerol backbone are important in this case. For example, 1,2-DAG isomers consistently delay polymorphic transitions more effectively than 1,3-isomers due to their crystallization characteristics (Hernqvist and Anjou, 1983; Oh et al., 2005; Smith and Povey, 1997; Wähnelt et al., 1991; Wright and Marangoni, 2002) and the effect of molecules with significantly different chain length is limited (Smith et al., 2007b; Smith and Povey, 1997; Wright and Marangoni, 2002). As the two isomeric forms were present it was difficult to assess their specific role. As the CB DAG were used in their originating substrate, the difference in chain length was limited. Hence, CB DAG and TAG co-crystallize and the DAG penetrate into the crystal lattice. The steric hindrance between the FA chains may hold and stabilize the hexagonal subcell packing structure of α forms, resulting in a retardation of the polymorphic transition to β' (Oh et al., 2005). This effect is similar to what is described by Schlichter-Aronhime et al. (1988) as the button syndrome:

surfactants have the capacity to create hydrogen bonds with the neighbouring TAG and so are able to control the polymorphic transformation.
At a certain moment during the isothermal period, α crystals started transforming into β'. Because of this, the supersaturation for the α polymorph increased again, allowing extra melt to crystallize in the α polymorph form. The so formed α crystals, also transformed into β'. In this case transition was solid mediated. However, this process is only temporarily as the samples up to 2.5% didn't show an isosbestic point in SAXS diffraction patterns. After a certain isothermal time β' crystallization still proceeded while all α crystals were already transformed. So the β' crystals could only originate from the melt.

For the samples with 5% and 10% DAG, the SAXS diffraction patterns showed a clear isosbestic point during the isothermal period indicating that the polymorphic transition from α to β' and the crystallization from the melt in the α polymorph occurred simultaneously (Dewettinck et al., 2004). In comparison to the other samples, the β' crystallization was thus only α-mediated. Two possible hypotheses can be postulated to explain this phenomena:

- The amount of α crystals was considerably higher compared to the samples with lower amounts of DAG, so the crystallization was preferably α mediated.
- Within the time frame of the experiment, the liquid mediated transformation to β' was limited. Smith et al. (2007b) have shown that DAG retard the rate of exchange between solid and liquid TAG. They claimed that crystallization happens at specific surface hotspot where there is a highly defected surface structure. DAG are able to attach to these areas and co-crystallize through incorporation of their chains into growing TAG crystals. However, the defective head group hinders further growth by setting a high steric penalty for being surrounded by TAG.

The rate constant K of the β' formation, determined by the Foubert model fitting, showed a decreasing trend as function of DAG concentration. The latter described mechanism besides the delayed polymorphic transition, may account for this observation.

Another remarkable finding was the appearance of a diffraction peak around 4.58 Å in the WAXD patterns of 5 and 10% DAG in the pattern of the β' polymorph. This is a typical peak for the β_v polymorph. In the small angle region, no changes were observed. Campos et al. (2010) observed a similar peak at 4.58 Å in their follow-up of CB crystallization in the presence of SSS and LLL at 20°C. This peak appeared after two days of storage, grew at the expense of the peaks 4.36 and 4.13 Å and no significant changes were observed in the long spacings. Longer storage revealed that a β' to β transformation was taking place. Based

on these finding, it can be suggested that some β crystals were formed. This can be due to the fact that 1,3-DAG isomers have a preference for the triclinic packing. It is possible that CB TAG are thereby forced to also transform to their more efficiently packed β crystals. A follow up with XRD over a longer isothermal period is necessary to further elucidate this phenomenon.

It is clear that the crystallization kinetics were affected by the DAG. When impurities, in this case DAG, are incorporated into a crystal they usually change the growth of a certain face preferentially, which results in changes in morphology (Wright and Marangoni, 2002). This may explain the differences that were observed when microstructure was visualized by PLM.

5.5 Conclusions

In this study a multi-methodological approach was used to study the isothermal crystallization, of cocoa butter in the presence of maximum 10% cocoa butter diacylglycerols over a period of four hours. At an isothermal temperature of 20°C, CB crystallizes in a two-step process: the α crystals formed during the first step transform to β' in the second step. DSC, pNMR, oscillatory rheology, XRD and PLM were used for this purpose. The high-melting disaturated DAG changed the saturation conditions of the melt and acted as seed crystals. The induction time of the first crystallization step was therefore inversely related to the amount of DAG. The subsequent polymorphic transition on the other hand was delayed by the presence of the DAG as they seem to stabilize the hexagonal packing. The good match in fatty acid composition between the DAG and CB may have contributed to this effect. Up to 2.5% DAG, besides solid-solid transition, β' crystals were also directly formed from the melt. At higher concentration the β' formation was only α-mediated. In future research it can be interesting to study the polymorphic behaviour over a longer storage period as when 5% or more diacylglycerols were present, the XRD diffraction patterns already showed a typical peak of the β form. This may indicate that the subsequent β' to β polymorphic transition may be induced by the presence of the diacylglycerols

Chapter 6

Functionality of cocoa butter diacylglycerols in chocolate

The Dawn of Woman

6.1 Introduction

The last years there is an increasing trend in chocolate manufacturing to more exclusive, complex and niche-oriented products where fillings, biscuits or wafers can be included. Many of these products consist of a thin chocolate shell with some kind of filling. These composed products lead to an increased processing complexity and a higher risk on quality issues of which fat bloom is the major concern in chocolate industry. Bloomed chocolate is characterized by the loss of its initial gloss and the formation of a grey-whitish haze, which causes the consumer to reject the product. A questionnaire addressed to the small Belgian chocolate producers, revealed that 57% is sometimes and 38% is frequently facing the fat bloom issue (21 respondents, representing a daily production capacity of 160 ton) (Depypere et al., 2009a). Although several studies have been performed to reveal the mechanisms, this phenomenon is not yet completely understood and so no optimal solution has been found to solve this problem.

As migration fat bloom is a major topic of this chapter, the following paragraphs give an overview of the mechanism of fat bloom, followed by oil migration and fat bloom formation in filled products.

6.1.1 Mechanism of fat bloom formation

Timms (2003) defined fat bloom as a new phase in a chocolate fat. This new phase becomes visible at the surface, often as clusters of large (5 μm), frequently whitish, crystals at several discrete points on the surface. It is a bulk phenomenon, usually observed first at the surface. This new phase can develop for three reasons:

i. A polymorphic change
ii. A transition from a single solid phase to two phases: liquid + solid
iii. A transition from a single solid phase to two solid phases

Although each mechanism is different, they often occur together and it is often not possible to determine which is the most dominant in the formation of fat bloom

i. Bloom may appear due to *polymorphic transitions* from β_{IV} to β_V or from β_V to β_{VI}. The transformation of β_{IV} to β_V is what typically appears in a poorly tempered chocolate. The β_V to β_{VI} transition is the most common as the development of form β_{VI} must eventually happen because this is the most stable form of cocoa butter. Sato and Koyano (2001) distinguish two temperature dependent types of polymorphic transformations. At low temperatures (<22°C) the high solid fat content results in solid-state transformations as the nucleation and

growth of β_{VI} crystals proceeds in the interior of β_V crystal particles. The oil-mediated transformation is limited because of low liquid concentrations thus the formation of the long, needle like crystals is limited and fat bloom may not be observed. The oil-mediated transformations on the contrary occur at higher temperatures with lower SFC values (± 25°C). The crystal growth of form β_{VI} can be initiated by either spontaneous secondary nucleation in the liquid or by heterogeneous nucleation at the surface of β_V crystals and catalytic ingredients such as sugar particles. The growing β_{VI} crystals are exposed to the liquid and extend the needle axis to produce the large, slender crystals characteristic for fat bloom formation. These mechanism are also applicable for the transformation of III and β_{IV} to β_V. The larger β_V crystals cause fat bloom.

ii. When chocolate is subjected to an increased temperature, the formed liquid occupies a greater volume than the solid. The resulting overpressure forces the liquid out to the surface through fissures and pores in the chocolate. Thus the solid and liquid phase become physically separated and are unable to recombine to give the original equilibrium solid solution. A temperature drop causes the liquid fat to re-crystallize with a composition different from the bulk fat. At the surface the liquid TAG crystallize into the most stable form and bloom occurs. Researchers often use temperature cycling to accelerate the rate of bloom formation. The continuous high to low temperature (and vice versa) change causes the liquid to be pumped to the surface. In addition, the higher temperatures induce the β_V to β_{VI} transition so that both mechanisms (i and ii) are associated (McCarthy et al., 2003; Timms, 2003).

iii. The *solid to solid + solid phase change* is mainly applicable for cocoa butter in combination with a lauric-type or a high-trans type of fat. From their phase diagram it can be derived that at certain conditions a two-solid phase system appears at equilibrium. Because it often takes several weeks or months to reach equilibrium, it is possible to make a compound chocolate to mould or enrobe with an acceptable appearance, snap and organoleptic properties. However, after several weeks, the system moves inevitably to equilibrium; the initial single solid phase changes to two solid phases and bloom appears. A more detailed explanation with several examples can be found in Timms (2003).

6.1.2 Fat bloom in filled chocolate products

6.1.2.1 Oil migration

Fat migration is a common problem in filled chocolate products with nut based centers, chocolate coated biscuits, cream filled chocolates and praline filled chocolate shells (Smith et al., 2007a). These products tend towards chemical and thermodynamic equilibrium

because of the compositional difference between the chocolate shell and the center. The movement towards equilibrium is achieved mainly by the movement or migration of the liquid phases (Timms, 2003). The liquid fat from the filling migrates into the surrounding chocolate and at the same time, the fats from the chocolate migrate to the filling as illustrated in Figure 6.1. At room temperature, lipids present in the fillings usually have a greater liquid fraction than cocoa butter so they can migrate more easily into the coating (Lonchampt and Hartel, 2004). This entire process results in softening of the chocolate layer, hardening of the centre, redistribution of flavours and fat bloom, and these adversely impact on the consumer acceptability (Ghosh et al., 2002; Ziegler et al., 2004).

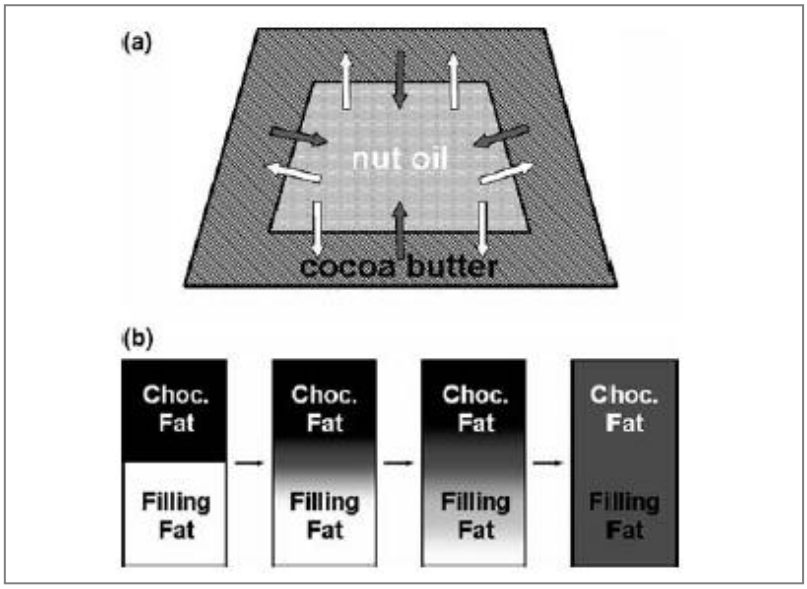


Figure 6.1 (a) Migration occurs from the centre to the shell and vice versa. (b) Given sufficient time, the composition of the fat phase in the center and the shell will become identical (Smith et al., 2007a)

Originally, the driving force for diffusion was assumed to be the difference in liquid fat content, but recently the diffusion has been attributed to a gradient in TAG concentration within some domains of the product. The migration of a molecular species occurs when there is a concentration difference of that particular species at that particular point; migration occurs until thermodynamic equilibrium. The movement of these molecules can be described by the principles of diffusion (Ghosh et al., 2002). The TAG molecules diffuse by random molecular motion to eliminate the concentration gradient or the difference in TAG composition between the oil present in the filling and the cocoa butter in the chocolate shell. A schematic representation is given in Figure 6.2. The theoretical aspects of oil migration are extensively described by Ghosh et al. (2002), Aguilera et al. (2004) and more recently reconsidered by Galdamez et al. (2009). Molecular diffusion is often used as a general model for mass transfer because an effective diffusion coefficient, D_{eff}, can be easily

determined from plotting the migrated mass logarithmically as function of time (Aguilera et al., 2004). The diffusion process can be described by Fick's law giving the impression that it's a single transport mechanism (Ziegleder et al., 1996a). However, diffusion is coupled with phase or structural changes that occur due to the presence of migrating molecules so non-Fickian behaviour will be observed. So when studying diffusion, it's important to couple the diffusion process with the microstructure and the phase behaviour (Aguilera et al., 2004; Ghosh et al., 2002). Therefore, Galdamez et al. (2009) proposed a predictive model based on combining molecular diffusivity and internal microstructure of chocolate.

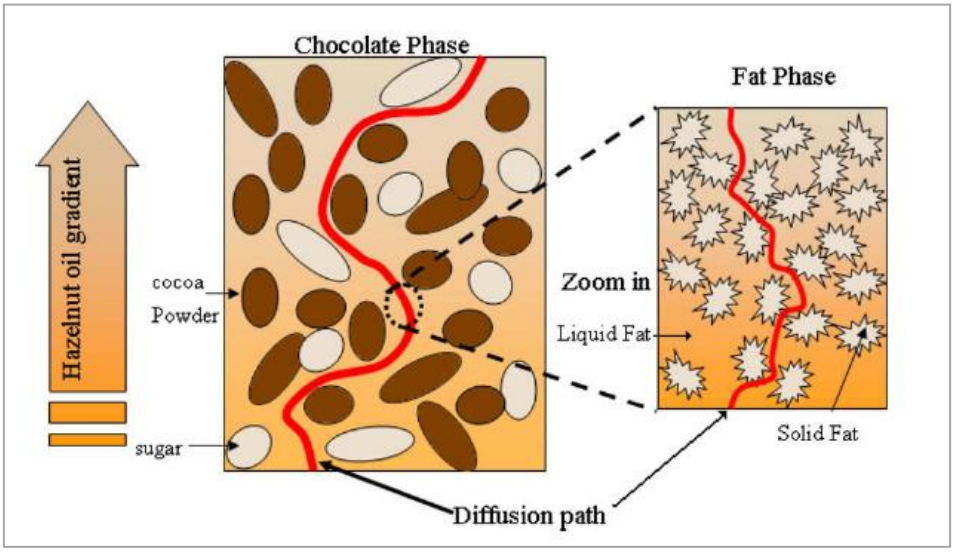


Figure 6.2 Schematic representation of hazelnut oil migration into chocolate (Galdamez et al., 2009)

Ziegler et al. (2004) stated that there is a link between the diffusion process and the phase behaviour. When nut oil from the filling diffuses into the surrounding chocolate shell, cocoa butter crystals will dissolve in the migrating liquid oil. Oil migration leads to a decrease in SFC of the chocolate shell, resulting in an increase in diffusion, leading to a further decrease in SFC which in turn increases diffusion in a kind of feedback loop. The question is whether diffusion of the oil throughout the liquid phase or dissolution of the solid phase is the rate limiting step. According to Ziegler et al. (2004), there seems to be a change in rate limiting mechanism between 20 and 23°C. At low temperatures (< 20°C), the chocolate is mostly solid and little dissolution occurs. In this case, diffusion dominates. At high temperatures (> 23°C), the fat is largely liquid and again diffusion dominates. In between these temperatures, the dissolution is rate-limiting.

Capillary pressure has been proposed as an alternative mechanism of oil migration (Aguilera et al., 2004; Guiheneuf et al., 1997; Lonchampt and Hartel, 2004; Marty et al., 2005; Walter

and Cornillon, 2002). In chocolate products, the liquid fractions are likely to move under capillary forces through defect structures such as interparticle pores, crevices, cracks or holes (Aguilera et al., 2004; Ghosh et al., 2002). The most common expression for capillary rise is the so-called Lucas-Washburn equation. This equation predicts that for short times the capillary rise (height of liquid column) should be proportional to the square root of time, which happens to be the same time dependence of a diffusion-controlled process. However, capillarity curves obtained after solving the Lucas–Washburn equation predicted an almost 800 times shorter duration for oil migration as compared to those observed experimentally, thus indicating little role of capillary forces in oil migration (Altimiras et al., 2007).

The exact mechanisms of oil migration are not well understood and most probably different mechanisms complement each other. Observation by magnetic resonance imaging by Deka et al. (2006) have shown that the dominant mechanism is diffusion and that if capillary pressure is involved it is a minor contributor.

The rate of migration of liquid TAG may be influenced by numerous factors: e.g., temperature, hardness of the fat, the particulate structure, emulsifier interactions and the porosity of the chocolate (Lonchampt and Hartel, 2004). A more detailed review is given by Ghosh et al. (2002).

6.1.2.2 Oil migration and the occurrence of fat bloom

The appearance of fat bloom on the surface of the chocolate is one of the main quality defects in filled chocolate products. Oil migration sets up the conditions necessary for fat bloom (Ziegler et al., 2004). These are two separated but correlated phenomena as oil migration is leading to a greater tendency towards bloom formation (Ghosh et al., 2002; Smith et al., 2007a). Oil migration is rather rapid whereas bloom formation may take much more time (Ziegler et al., 2004).

Liquid fat from the filling migrates into the chocolate where it mixes with the cocoa butter where they behave according to their phase behaviour. As a result, the solid fat content of the coating decreases due to dilution or dissolution of the cocoa butter crystals in the liquid fat. The liquid TAG with greater mobility are likely to crystallize into a more stable polymorph and visible bloom occurs (Lonchampt and Hartel, 2004).

According to Ziegler et al. (2004), migration bloom occurs due to solvent-induced re-crystallization via the mechanism of Ostwald ripening. The solubility of smaller crystals in liquid oil is greater than that of larger crystals. At temperatures above the melting point of the smaller crystals, but below the melting point of larger crystals, the smaller crystals melt and are deposited onto the larger crystals, leading to the growth of the larger crystals. Furthermore, re-crystallization also occurs through accretion, i.e. fusion of small crystals to

form larger crystals. This would eventually lead to a chocolate with only a few large crystals and a very coarse texture. If the formation of needle-like structures at the surface is limited, no visible fat bloom will appear on the chocolate. Lonchampt and Hartel (2004) mention that aluminum foil in contact with the chocolate surface retards fat bloom formation. The foil hinders the crystals to grow and protrude from the chocolate surface by which no visible fatbloom will be detected.

The hypothesis of fat bloom development, as suggested by Ziegleder (2006) states that the filling fat migrates to the chocolate but there is very few migration in the opposite direction. This leads to a surplus of fat in the chocolate coating. The fat mixture, consisting of filling fat, liquid cocoa butter and dissolved cocoa butter, further migrates to the chocolate surface where it can crystallize. β_{VI} crystals accelerate the growth of bloom due to the needle shape. There is a clear correlation between fat migration and the onset of fat bloom, but there is no correlation with the growth of bloom. The latter is determined by the composition of the migrating oils.

Ziegleder et al. (2001) demonstrated that migration encourages polymorphic transitions and the polymorphic transition to β_{VI} encourages migration. According to Smith et al. (2007), hazelnut oil promotes the transition from β_V to β_{VI}. Even small amounts of hazelnut oil (1%) can cause polymorphic transition.

The storage temperature also plays a major role in the formation of fat bloom. At higher temperatures, there is not only an increase in oil migration, but also in polymorphic fat bloom. However, migration fat bloom is reduced from 23°C onwards (Ziegleder and Schwingshandl, 1998). This can be explained by the combination of a higher solubility and the impossibility to crystallize. The occurrence of migration fat bloom can be ordered as follows:

$$20°C > 23°C > 18°C \approx 26°C > 10°C.$$

An “optimum” temperature for bloom formation has been defined: 18 – 22°C for milk chocolate and 18 – 26°C for dark chocolate (Timms, 2003).

6.2 Research strategy

In the first part of this research, the cocoa butter based diacylglycerols were applied in a dark chocolate formulation up to 25% on fat base and the functional properties were derived by defining melting and flow behaviour and texture.

In Chapter 5 it was observed that minor amounts of DAG had an influence on crystal network formation and so on microstructure. Differences in crystallization behaviour during,

e.g., tempering may therefore lead to the formation of different microstructural organization of the crystal network, resulting in physical changes in the chocolate.

Moreover MAG, DAG, monoglycosyl DAG and sucrose fatty acid ester have all been proposed as bloom inhibitors for compound coatings (Lonchampt and Hartel, 2004). More specifically, the Japanese patent 05168412 (Gunji et al., 1993) describes the use of DAG as bloom preventing agents.

The development of bloom in filled chocolates depends upon the interaction between liquid oil and the solid fat. Smith et al. (2007b) have demonstrated that MAG and DAG have a retarding effect on the rate of exchange between solid and liquid phases. In Chapter 5 it was also seen that at 5 % and 10% DAG, an isosbestic point was observed, indicating that β' formation was only α-mediated.

Consequently, based on the above described findings, it seemed of interest to evaluate the appearance of fat bloom in the produced DAG chocolates. Studies on migration fat bloom require a setup in which chocolate is brought into contact with a filling. A model system consisting of a cylinder filled with filling and chocolate was utilized for this purpose. Chocolates without filling were used as control samples. The surface of the chocolate was examined at regular time intervals to detect the presence of fat bloom crystals. The surface of the chocolate was assessed by an automated image analysis procedure. The chocolate was cut into different layers and analyzed by HPLC and DSC to monitor the oil migration.

6.3 Materials and methods

6.3.1 Samples

A standard dark chocolate with 25% fat (Barry Callebaut, Wieze) was adjusted to 35% fat by adding CB or a mixture of CB and DAG so that chocolates with different amounts of CB DAG, 1.25 % – 2.5 % – 5 % – 7.5 % – 10 % – 12.5 % – 25 % on total fat base, were obtained. On a product base the amount of DAG varied between 0.4% and 8.8 %.

To evaluate migration fat bloom in this setup, a hazelnut filling was produced by Barry Callebaut (Wieze, Belgium). The hazelnut base was prepared by mixing 50% sugar with 50% hazelnuts. A standard dark chocolate was added in a ratio of 25/75 chocolate/hazelnut filling. Model systems were produced in small cylinders (height: 5 cm, radius: 2 cm) by adding a 0.5 cm chocolate layer on top of a 3.5 cm filling layer. The different samples were stored at 20 °C and monitored for 1 year by analyzing three samples on a 2 to 3 weekly basis.

6.3.2 Tempering

As it was difficult to predict the influence of the DAG on the tempering properties of the chocolate, it was decided to temper by hand on a marble table. Tempering was performed by a skilled chocolate confectioner of Barry Callebaut. Similar to traditional tempering 2/3 of the liquid chocolate at 40 – 45°C was poured on the marble table and by manual handling cooled to ± 28 – 27°C. The cooled chocolate was mixed with the hot chocolate, well stirred to homogenize the temperature and to melt the unstable crystals. After tempering, the chocolate was poured in the hopper of the One Shot machine (Knobel, Felben b. Frauenfeld, Germany) to automatically fill the cylinders (with or without filling).

6.3.2.1 Rheology

To measure the flow behaviour of the produced chocolates a TA Instruments AR2000ex (TA Instruments, New Castle, Delaware, USA) with plate-plate geometry was used. The temperature of the bottom plate was set to 40°C. A small amount of chocolate was applied on the plate. A stepped flow procedure was applied by increasing the shear rate logarithmically from 0.01 s^{-1} to 100 s^{-1} while measuring the shear stress. Every chocolate sample was measured in triplicate. The Casson-model was fitted to the resulting correlation between shear rate (from 0.1 to 65 s^{-1}) and shear stress.

6.3.2.2 Texture analysis

A three point bend test was performed on a TA500 Texture Analyzer (Lloyd Instruments Ltd., West Sussex, UK) equipped with a 500N load cell and ball probe. The fracturability is the maximum load [N] necessary to fracture a bar of tempered chocolate. The probe descended at 10 mm/min until the chocolate bar cracked. For every test, ten repetitions were performed.

For the penetration test, a cylindrical probe (diameter 4.51 mm) descended in a chocolate bar with a speed of 10 mm/min over a distance of 5 mm, the measurements starts at a trigger of 0.2 N. The maximum load is defined as the hardness. For every test, 10 repetitions were performed.

6.3.3 Migration fat bloom

The model systems (cylinder with filling and chocolate) were produced for six different chocolates: two reference chocolates without DAG, 1.25%, 2.5%, 5% and 7.5% replacement of CB by CB DAG. On a product base, the amount of DAG varied between 0.4 and 2.6%. In

parallel, cylinders with only chocolate were used as a blank to be sure that the observed effect was due to migration and not to changes occurring in the chocolates over time.

6.3.3.1 Image analysis procedure

The image analysis algorithm developed by Nopens et al. (2008) was used to evaluate fat bloom. Every sample was photographed in double. Each picture showed a circle representing the surface of the chocolate sample on a whitish background. The collected pictures were analyzed using an image analysis procedure developed in the IMAQ Vision builder (National instruments, USA). The TIFF format pictures with a 24-bit RGB colour format were converted into the HSL-scale (Hue, Saturation, Luminescence). In this scale luminescence represents the picture in an 8-bith resolution grayscale (0 – 255). To detect the chocolate on the white background of the picture an edge detecting algorithm, a spoke, was used. By using a mask function, the region of interest was extracted and further analyzed. In the analysis a histogram was calculated based on the grayscale pixel formation. The size of the circles was decreased by reducing the number of pixels. More detailed information about the image analysis set up and processing procedure for the pictures can be found in Nopens et al. (2008). In this study, four values were chosen: -35; -110; -150 and -200 pixels. In order to compare histograms of several samples, a mean pixel value was calculated as the summarizing parameter of one histogram.

6.3.3.2 Melting behaviour

The surface of the chocolate was scraped off with a scalpel and 5 – 10 mg of the chocolate flakes were transferred to an alodined pan for subsequent analysis in a 2010 CE DSC with refrigerated cooling system (TA Instruments, New Caste, USA). When the system reached the equilibrium conditions at 20°C, the pan was put in the DSC cell and the melting profile was recorded by heating at 5°C/min to 65°C.

The melting curves were integrated with a linear baseline using Universal Analysis 2000 software (TA Instruments, New Castle, USA). The end point was always fixed at 40°C. The starting point was determined as the intersection of the linear baseline and the beginning of the melting curve in such a way that the baseline was close to being horizontal. As there was often a lot of noise at the beginning of the melting curve, it was decided to perform a perpendicular drop at 26°C. Results were considered from this point to have a reproducible integration permitting to compare the melting behaviour of the different samples.

Following parameters were extracted from the melting curve:

- *Peak maximum* (°C): the temperature at which the crystallization or melting curve reaches its peak maximum
- *Peak area* (J/g): the heat taken up by the sample during melting, this was calculated by integrating the area of the melting peak
- *Onset temperature* (°C): determined by the intersection of the baseline with the absolute highest tangent of the melting curve
- *Width at half height* (°C): indication for the width of the melting peak

Besides the surface, also the melting behaviour of the different layers (see next section 6.3.3.4) was studied by applying the same melting protocol.

6.3.3.3 Scanning electron microscopy

A small piece of chocolate was sputter-coated with gold during 60s using a JEOL JFC and observed with a JEOL JSM-5600 scanning electron microscope (Jeol, BV Zaventem, Belgium) operated at 10kV.

6.3.3.4 Sampling procedure

A microtome is a sectioning instrument that allows the cutting of extremely thin slices of a sample. To monitor the migration of the filling fat through the chocolate, the disks were removed from the filling and cut in different layers by a LKB Bromma 2218 Historange microtome. The chocolate disk was glued on the sample holder and different layers were cut by the gradually approaching knife. The distance was set at 5 μm and 10 cuts were performed per layer. As the chocolate disks had a thickness of 0.5 cm it was expected to collect 10 layers. However, in practice only 8 layers were obtained due to the varying thickness and the loss of chocolate due to the gluing. Layer 1 (filling side) was saturated by the filling so the first layer that was taken into consideration was layer 2. At sampling, 3 samples were randomly selected. The surface was first analyzed by taking a picture and by scraping some chocolate from the surface to record a DSC melting profile. The chocolate was then removed from the filling and the different layers were then subsequently analyzed by DSC to record their melting profile. The fat phase was extracted prior to TAG analysis. A schematic representation of this set up is given in Figure 6.3.

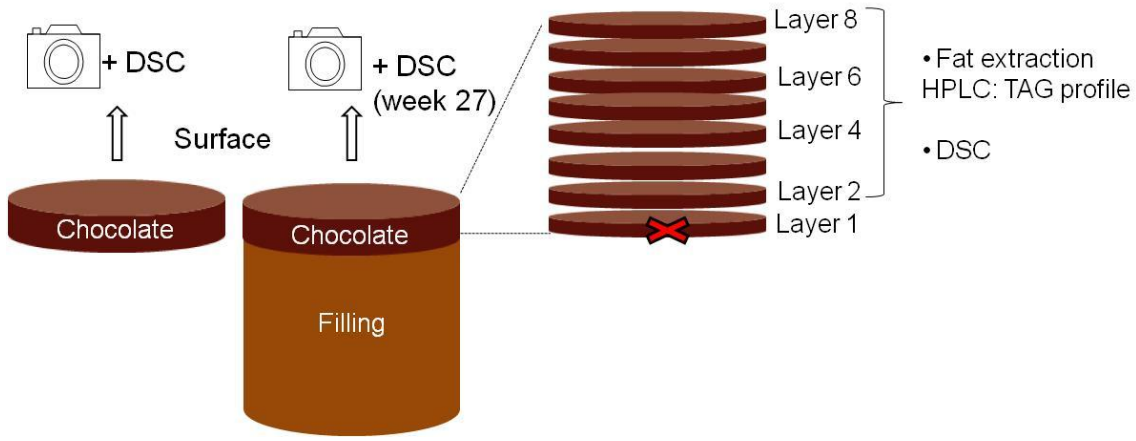


Figure 6.3 Schematic representation of the different layers obtained from the model system and the analyses which were performed

6.3.3.5 Fat extraction method and TAG distribution

In order to assess the degree of migration of the filling oil to the chocolate during the storage period, the TAG composition of the different layers was analyzed. For that purpose, the fat was extracted from the chocolate matrix. The chocolate sample was dissolved in 10 ml of petroleum ether, vigorously shaken and centrifuged (10 min, 3000 rpm). The solvent of the supernatant was evaporated at 50ºC.

The remaining fat was dissolved in the mobile phase, acetonitrile/dichloromethane 70/30 (v/v) for the subsequent HPLC analysis. TAG profile was analyzed by the method described by Rombaut et al. (2009). This method is described in more detail in section 3.3.7. Peak areas were correlated with the surface areas of the different TAG in the sample. Per sampling time three samples were analyzed in double.

6.4 Results and discussion

6.4.1 Influence of CB DAG on chocolate quality attributes

The DAG characterized in the previous chapter were used in the production of the chocolate samples as described in Section 6.3.1. In this chapter, the % DAG always refers to the replacement on fat base. As it was difficult to predict the influence of the DAG on the tempering properties of the chocolate it was decided to temper by hand on a marble table. It was observed that the chocolate with 10% CB replacement showed more thickening, indicative for the rapid nucleation of the higher melting DAG. After tempering, the chocolate was poured in the hopper of the One Shot machine to automatically fill the cylinders (with or without filling). The temper index before filling varied between 1.2 – 2.6 for the different chocolates. A higher TI and thus a higher degree of pre-crystallization, tended to block the

nozzles of the machines. After filling the TI increased to values between 3 – 4. For chocolates with 12.5 % and 25 % replacement, the temper meter did not give a value probably due to the aberrant crystallization behaviour.

6.4.1.1 Melting behaviour

The chocolates were stored at 20°C and sampled every three weeks to record a melting profile. Sampling started one week after production. The DSC melting profiles were analyzed to derive the melting characteristics as described in section 6.3.3.2. The results of two representative chocolates, chocolates with 0% and 5% CB replacement, are presented in Figure 6.4. It can be observed that peak area, peak maximum and onset reached equilibrium values beyond four weeks storage. The width at half height remained stable during the first month. Differences between the two chocolates were significant after one week storage but disappeared upon prolonged storage. The increasing peak area pointed out that during storage post crystallization occurred: more solid fat resulted in higher melting enthalpies. Addition of DAG seemed to slow down this post crystallization process. The peak maxima at week 1 were still quite low, indicating that β_{IV} crystals were present. Therefore, the shift to higher peak maxima was probably the result of the re-crystallization to β_V.

The melting profiles of the different chocolates after four weeks storage are presented in Figure 6.5. The corresponding DSC parameters are presented in Table 6.1. The melting profile of chocolate is characterized by a sharp, narrow melting range resulting in a quick melt down at body temperature. This typical behaviour produces a cool sensation and it is responsible for the pleasure release of flavours (Schlichter-Aronhime and Garti, 1988). Up to 7.5% replacement, the influence was limited as peak maximum and area were not significantly different. However, at 7.5% a small peak appeared around 42°C, due to the fractional melting of the high-melting DAG. For 12.5% and 25% DAG this peak increased and shifted to higher melting temperatures. This will give the chocolate a waxy mouthfeel upon tasting. The peak maximum and onset temperature of 12.5% and 25% were significantly lower compared to the other chocolates. The melting peak was also broader resulting in a significantly higher peak width (Table 6.1). These differences persisted upon longer storage. It could be concluded that up to 7.5% addition, the melting behaviour did not significantly change compared to the reference chocolate without DAG.

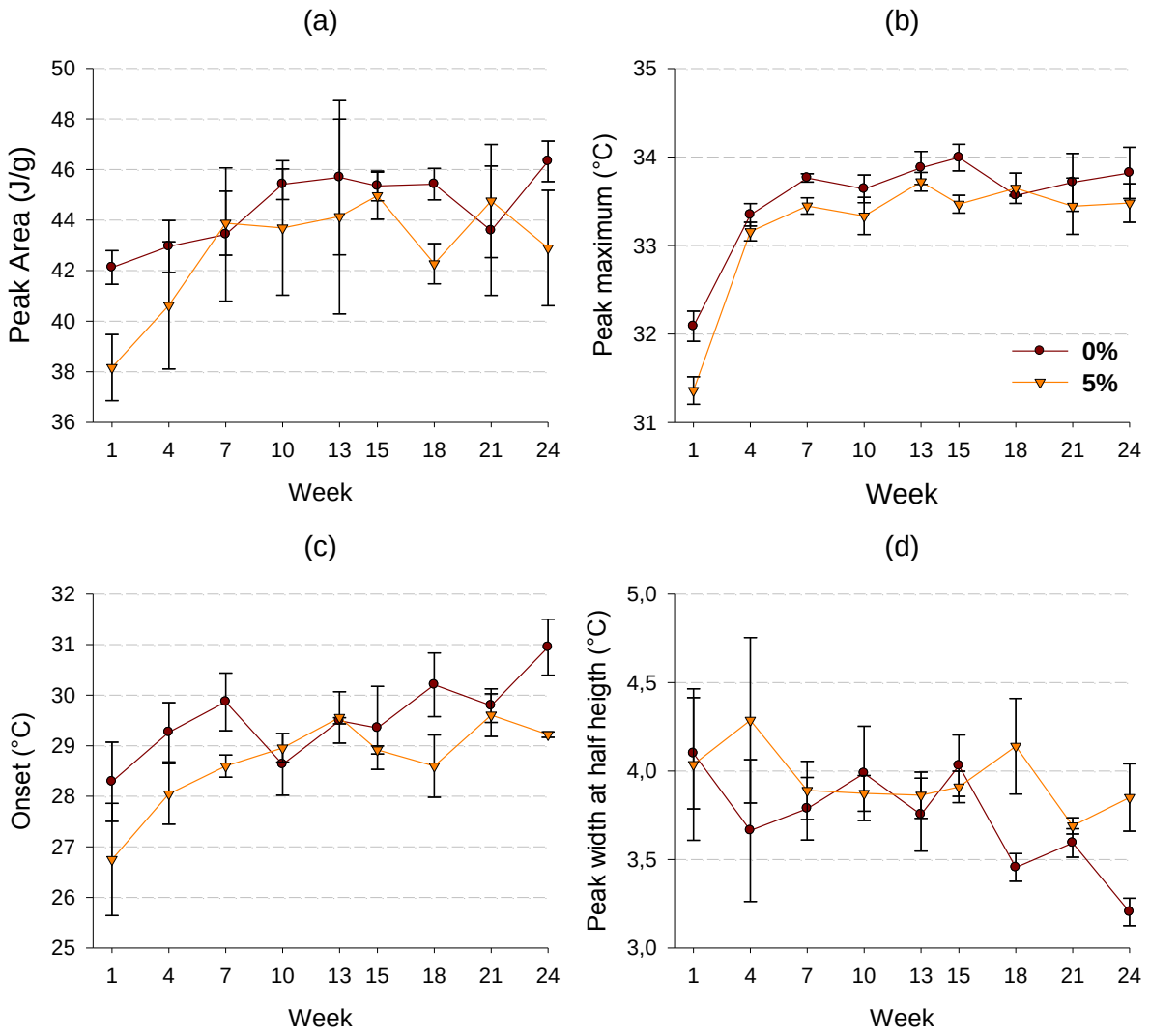


Figure 6.4 Peak Area (a), peak maximum (b), onset (c) and peak width at half height (d) for two chocolates with 0 % (●) and 5% (▼) DAG

Table 6.1 Peak maximum and melting enthalpy after four weeks of storage.

% DAG	Peak maximum (°C)	Peak Area (J/g)	Onset (°C)	Width at half height (°C)
0	33.35±0.13	42.95±1.03	29.27±0.59	3.66±0.40
1.25	33.39±0.26	42.24±1.32	28.67±0.72	3.87±0.46
2.5	33.22±0.07	42.28±4.64	28.65±0.74	3.86±0.43
5	33.16±0.11	40.63±2.52	28.05±0.60	4.29±0.47
7.5	33.01±0.23	39.33±0.10	27.92±0.20	4.19±0.10
12.5	32.10±0.29*****	40.60±0.30	26.40±0.34*****	4.91±0.23*****
25	31.17±0.58*****	41.09±1.10	24.54±1.55*****	5.18±0.23*****

*indicates significant difference (α=0.05)

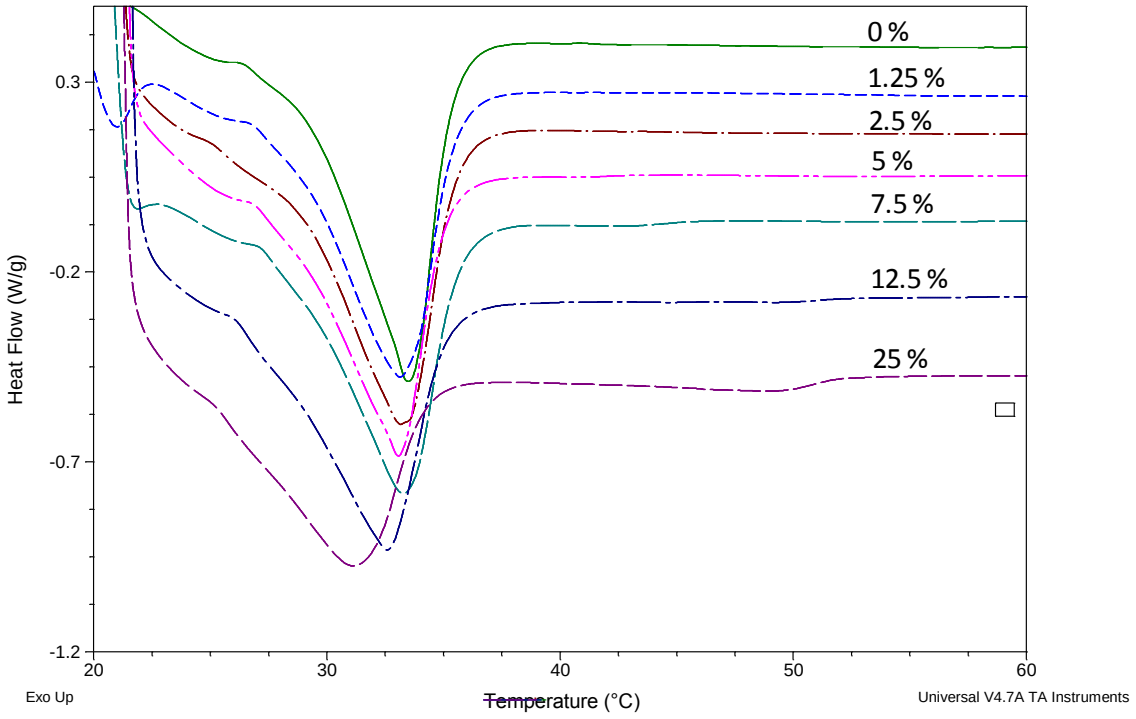


Figure 6.5 Melting profiles after four weeks of storage of tempered chocolates with varying amounts of DAG (on fat base)

6.4.1.2 Flow behaviour

The flow curves of the chocolates with increasing amounts of DAG are shown in Figure 6.6. In the measuring procedure, the temperature was set at 40°C. It can be seen that up to 5%, the flow curves tend to overlap, which is indicative for similar flow characteristics. Beyond 5%, the curves shift to higher shear stresses. For the chocolates with 25% DAG, an overshoot was observed at the lower shear rates which is probably due to some remaining high-melting crystals; in Figure 6.5, some remaining crystals at 40°C were observed. This residual crystal network was probably destroyed by applying shear resulting in a sudden increase of the shear stress. The Casson model was fitted to define the Casson yield stress and viscosity. This was not possible for the flow curve of the chocolate with 25% DAG. In Figure 6.7 the two Casson parameters were plotted as function of the DAG concentration. Up to 5% the yield stress and viscosity of the chocolates were similar: the DAG didn't influence the rheological properties of the chocolates. At higher concentrations the yield stress and viscosity showed an increase. DAG have emulsifying properties so they will interact at the interface together with the lecithin molecules to coat the sugar particles and the cocoa particles. Up to 5%, the DAG didn't influence the effectiveness of the phospholipids molecules of the lecithin. At higher concentrations, yield stress proportionally increased when more DAG were present in the chocolate. It is known that at high emulsifier

concentrations, an increase in yield value is linked to reverse micelle formation in the continuous phase or to the formation of multilayers around the sugar particles which hinder the flow (Afoakwa, 2010; Beckett, 2009a; Ghorbel et al., 2011). This may explain the observed increase in yield stress beyond 7.5% DAG. The increase in viscosity at the higher concentrations was probably due to the fact that at higher concentrations the high-melting fraction is not completely melted 40°C making it more difficult for the chocolate to flow, resulting in higher viscosity values. Viscosity measurements at higher temperatures should confirm this influence of a higher melting fraction on the viscosity.

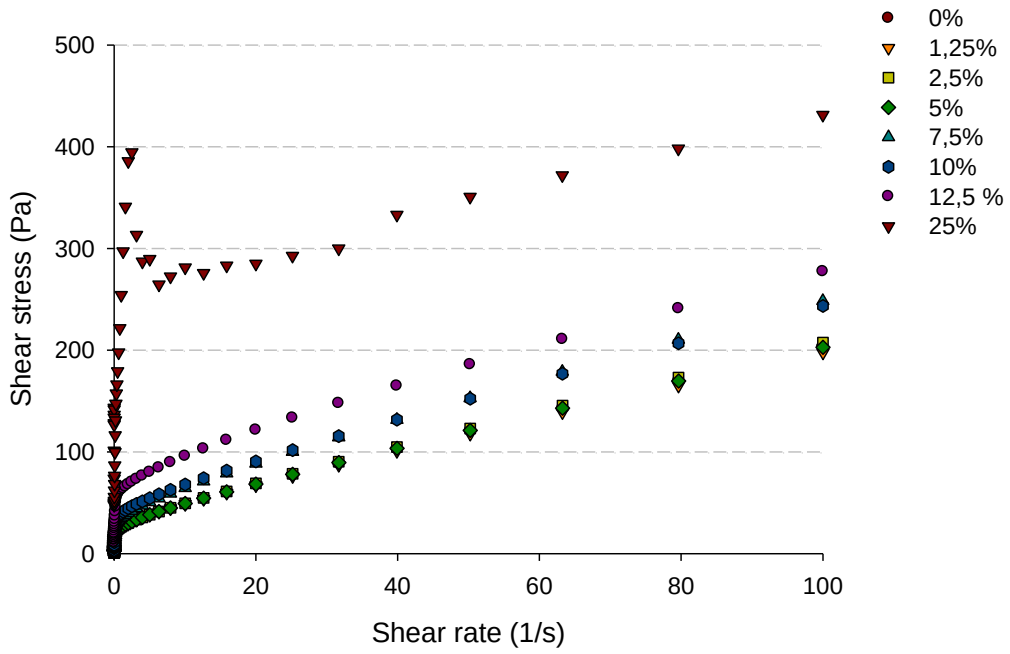


Figure 6.6 Flow curves of chocolates with different concentrations DAG (on fat base)

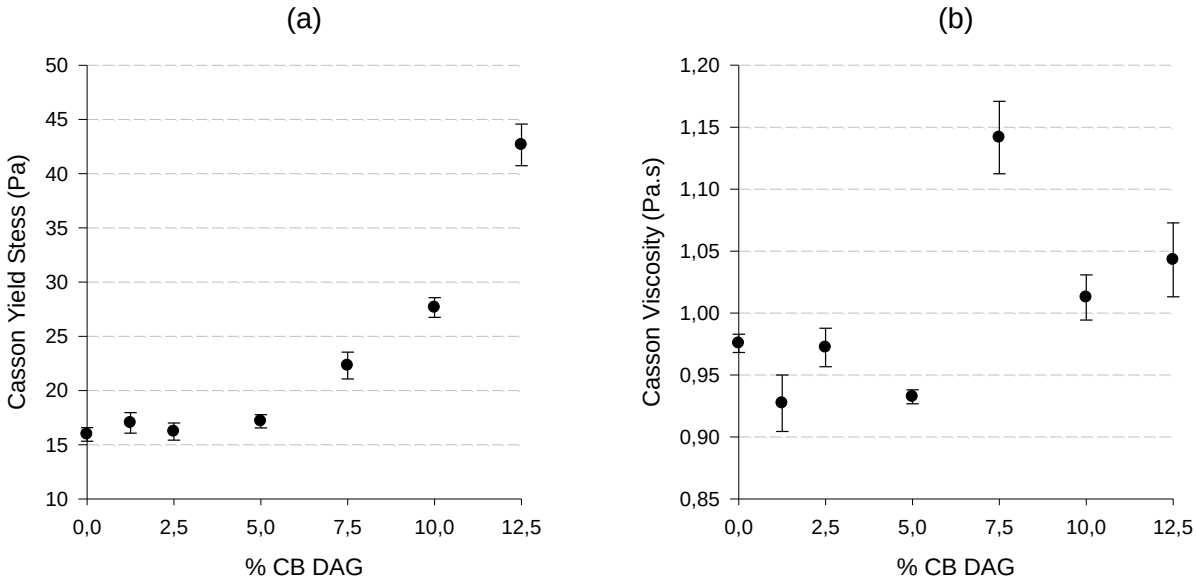


Figure 6.7 (a) Casson yield stress and (b) Casson viscosity as function of DAG concentration (on fat base)

6.4.1.3 Texture analysis

The desired characteristics for a chocolate are a firm solid product with a good snap at ambient temperature and a glossy appearance, combined with an easy melting in the mouth resulting in a smooth mouthfeel (Afoakwa, 2010). To evaluate the macroscopic properties, the fracturability and hardness were measured one week after production. These measurements were performed on tempered and untempered chocolate. Flexible moulds were used to be able to demould the untempered chocolate. From Figure 6.8 it can be derived that tempering clearly influenced both parameters. For the untempered chocolates up to 7.5% DAG, the fracturability was equal and almost half of the fracturability of their tempered counterparts. For 12.5% DAG, the difference between tempered and non tempered chocolate became smaller. Moreover, for the chocolate with 25% replacement, both fracturability and hardness were equal for the tempered and untempered chocolate. The DAG had a more pronounced influence on the hardness (measured by a penetration test): a linear increase was observed when more cocoa butter was replaced. As observed in the DSC profiles, chocolates showed post-crystallization beyond one week storage; therefore it can be expected that differences would decrease upon further storage.

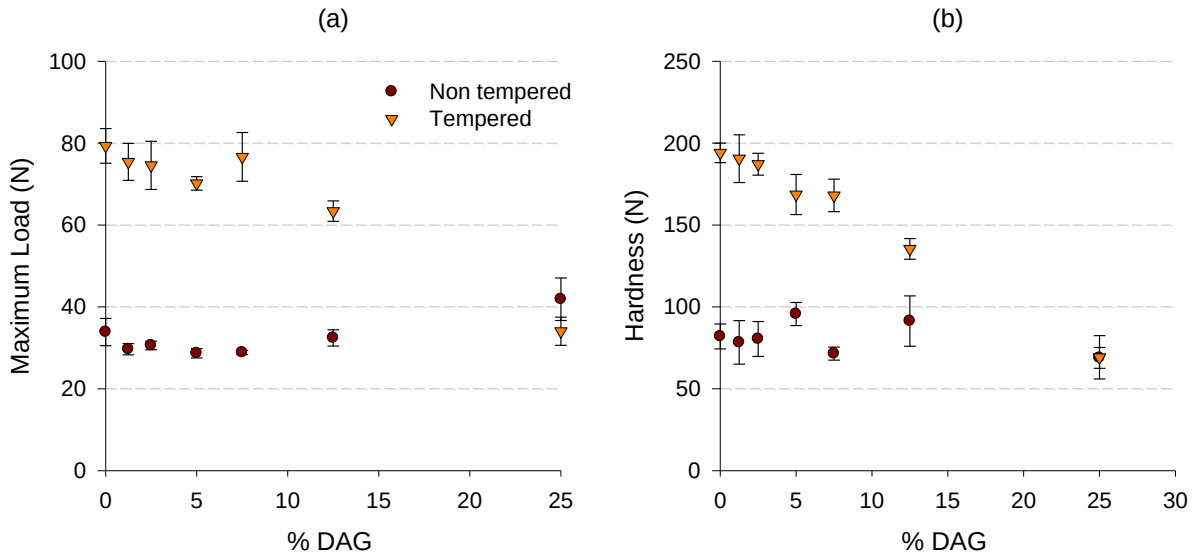


Figure 6.8 (a) Fracturability and (b) Hardness as function of DAG concentration (on fat base) after 1 week storage at 20°C

6.4.2 Influence of CB DAG on migration fat bloom

For this set up model systems were used as described in section 6.3.3. Five different chocolates were selected: a control chocolate without DAG, four chocolates containing 1.25%, 2.5%, 5% and 7.5% DAG on fat base (corresponding with 0.4 to 2.6% DAG on a product base). Samples without a filling were used as control. A schematic representation of this set up is given in Figure 6.3.

6.4.2.1 Visual assessment

At sampling time, three samples with and without filling were randomly selected. Firstly, a picture of the surface was taken. As stated by Nopens et al. (2008), two different aspects should be detectable due to the nature of fat bloom: (1) the disappearance of the gloss and (2) the appearance of a “whitish” area on the dark chocolate background. In Figure 6.9 some pictures are presented to show the evolution of their appearance as function of time. The first part (Figure 6.9a) presents the surface of the chocolate without extra DAG in contact with the filling. In the pictures of the first weeks (up to week 18) some reflection was visible due to the gloss of the chocolate. This is indicated with a red arrow on the picture of week 4. After 24 weeks, the chocolates had lost their gloss and as storage progressed the dark brown surface transformed to a lighter brown, gray like surface with some white spots.

The control sample, the chocolate without filling (Figure 6.9b) didn’t show this loss of gloss nor the appearance of a grey surface. Even after almost one year of storage, some reflectance, gloss was still present. Consequently, the effects observed in the filled chocolate

were due to the contact with the filling and not inherent to the chocolate itself. In the last row (Figure 6.9c), some selected pictures were presented for the chocolate with 7.5% DAG. Similar trends were observed compared to the reference: gloss disappeared and the surface turned dull with brown/grey colour.

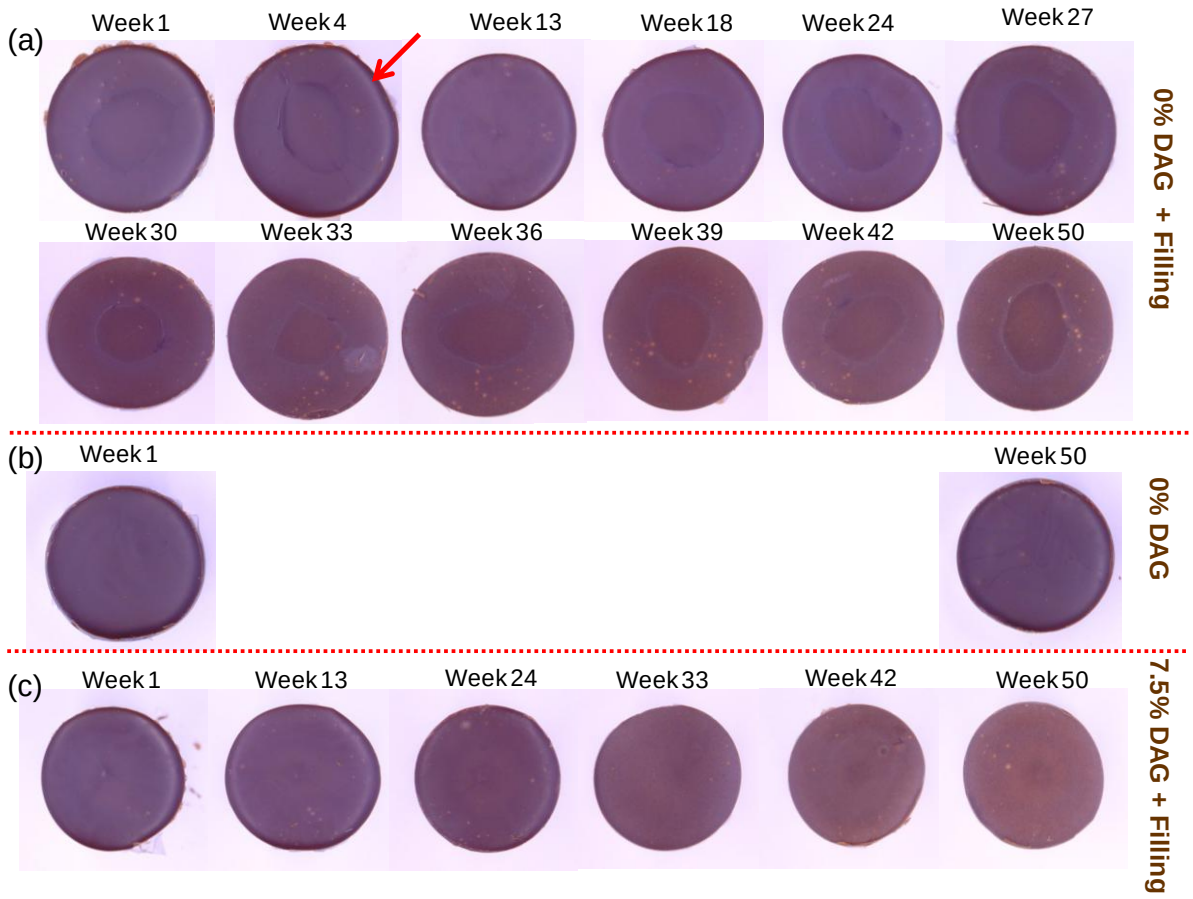


Figure 6.9 Overview picture of (a) Reference chocolate + filling (b) Reference chocolate and (c) chocolate 7.5 % DAG + filling at different sampling times

6.4.2.2 Image analysis

The image analysis method of Nopens et al. (2008) demonstrated to be a solid alternative for the panel procedure. Therefore pictures were processed with the algorithm developed by these authors. More details are described in section 6.4.2.2. A histogram was calculated based on the grayscale pixel formation. The size of the circles was decreased by reducing the number of pixels. Four values were chosen: -35, -110, -150 and -200 pixels. In order to compare histograms of several samples, a mean pixel value was calculated as the summarizing parameter of one histogram.

As described in the previous paragraphs, reflection faded out over time and the gloss disappeared. The occurrence of this reflection can by checked by analyzing a picture for

different circle radii. This should result in a change in the histogram when reflection is present, whereas no difference will be observed when reflection is not present (Nopens et al., 2008). These authors were able to determine the gloss disappearance. In this study it was very difficult to undoubtedly determine the disappearance of the reflection. Over the whole sampling period, the change in histogram remained equal when varying the different radii even though the surface was already dull.

The mean pixel value was always determined at the same radius of -110. This parameter was used to evaluate the “whitening” of the surface. Higher grayscale values indicate a “whiter” colour. The results of the picture analysis of the control chocolates and the chocolate in contact with filling are presented in Figure 6.10a and b respectively. For the chocolates without filling, the mean pixel value varied from 95 to 110 and remained within this interval during the whole storage period. For the chocolate with fillings, this mean pixel values also showed values between 95 – 105. Between week 18 and 30, there was a tendency that the mean pixel values were lower but from week 30 onwards a shift to higher values (110 – 115) was observed. Compared to the control samples a significant increase was observed. The higher mean pixel values indicated that the surface was “whiter” compared to the control samples. Nopens et al. (2008) observed a more pronounced increase: mean pixel values increased from values around 20 to 70. It should also be noticed that no significant differences were observed as function of the DAG concentration.

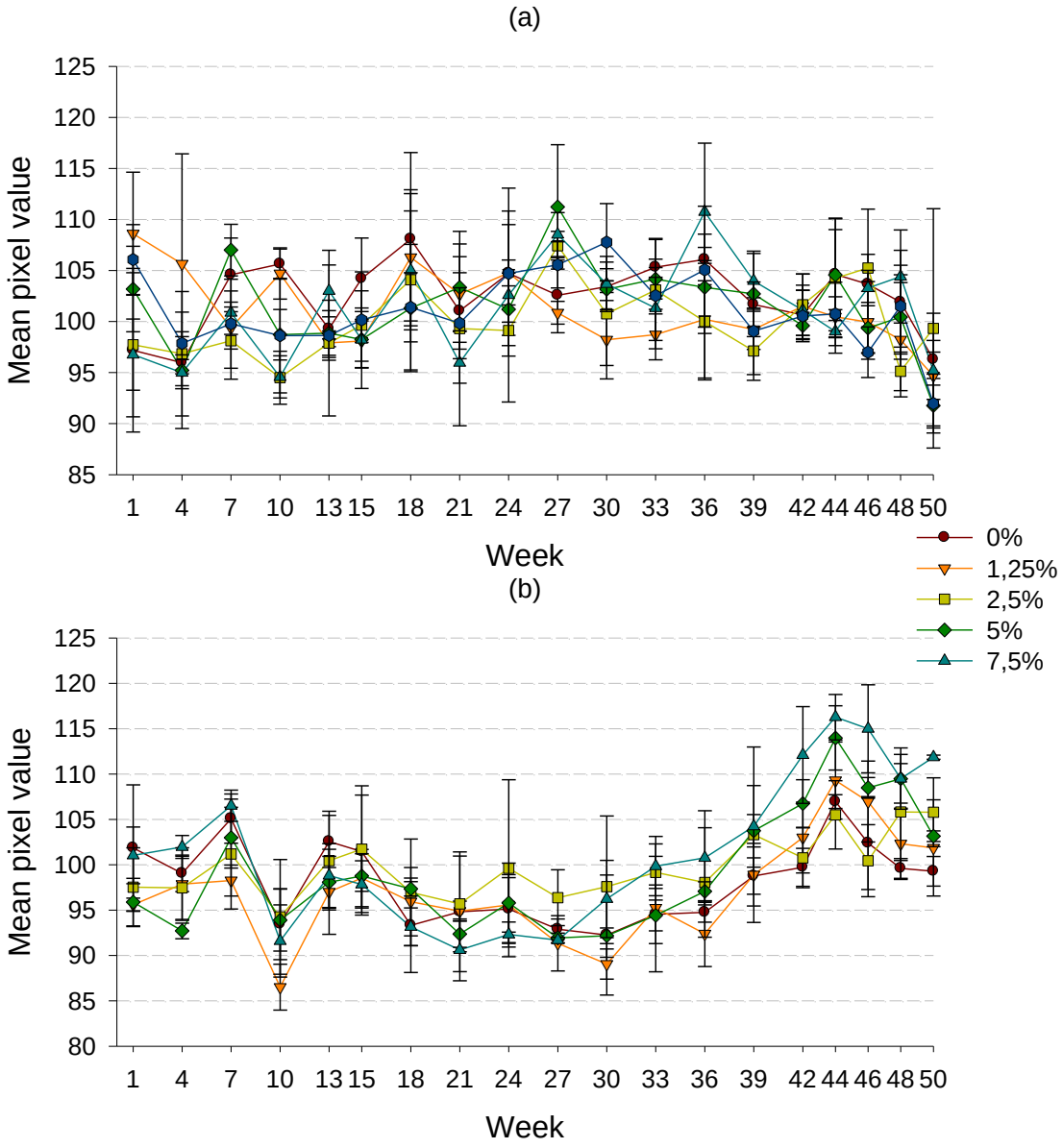


Figure 6.10 Mean pixel value for (a) chocolate without filling (b) chocolate with filling for the different storage times

6.4.2.3 Melting profile of the chocolate surface

In the next step of the sampling process, the surface was scraped off with a scalpel and analyzed by DSC to investigate the melting behaviour. This analysis was performed in the second half of the experimental set up. The melting characteristics (peak maximum, area, onset temperature and peak width) were derived from the melting curves. The results for the five different concentrations are presented in Figure 6.11 and Figure 6.12. The graphs on the left side (a and c) show the results of the control chocolates, without filling, and those on the right side (b and d) show the corresponding results for the chocolates in contact with the filing.

– After 27 weeks, the *peak maximum* (Figure 6.10a-b) was around ~33.5°C for the chocolate with and without contact with the filling. Beyond 33 weeks, the peak maximum of the chocolates with filling steadily increased to ~ 35°C to remain stable from week 42 onwards. For the chocolates without filling a limited increase was observed to end up around 34°C after 50 weeks of storage.

– The *peak area (*Figure 6.11c-d) didn't change as function of storage time for the control samples. Melting enthalpy ranged from 44 – 50 J/g. For the integration of the melting curve 40°C was always selected as endpoint. As some fractional melting of the high-melting DAG occurred above this temperature, the peak area of the chocolate with 7.5% DAG was consistently lower compared to the other samples. For the chocolate in contact with the filling a doubling of peak area was observed beyond 39 weeks of storage. This finding pointed out that the surface was enriched with pure fat crystals, protruding through the chocolate surface.

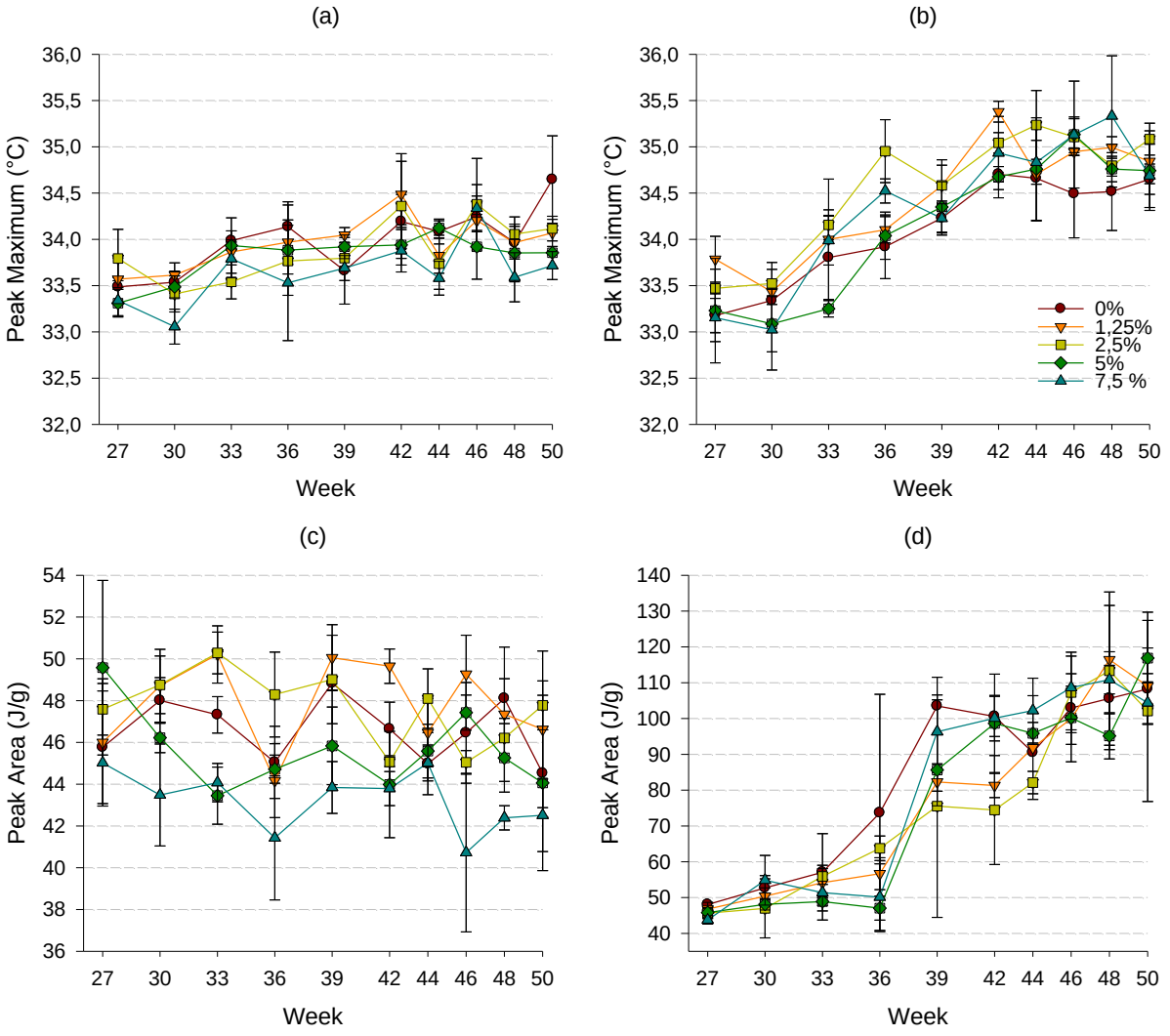


Figure 6.11 Peak maximum and peak area as function of sampling time for the control chocolate (a,c) and chocolates with filling (b,d)

For the control chocolate, *the onset temperature* (Figure 6-12a-b) didn't change as function of storage time (~29-30°C). The chocolate with filling on the other hand showed a linear increase in the onset temperature as function of the storage time to obtain a constant value around ~33°C from week 42 onwards.

- During storage the *peak width at half height* (Figure 6-12c-d) of the control samples became slightly broader while for the samples with the filling the melting peak became more narrow upon storage.

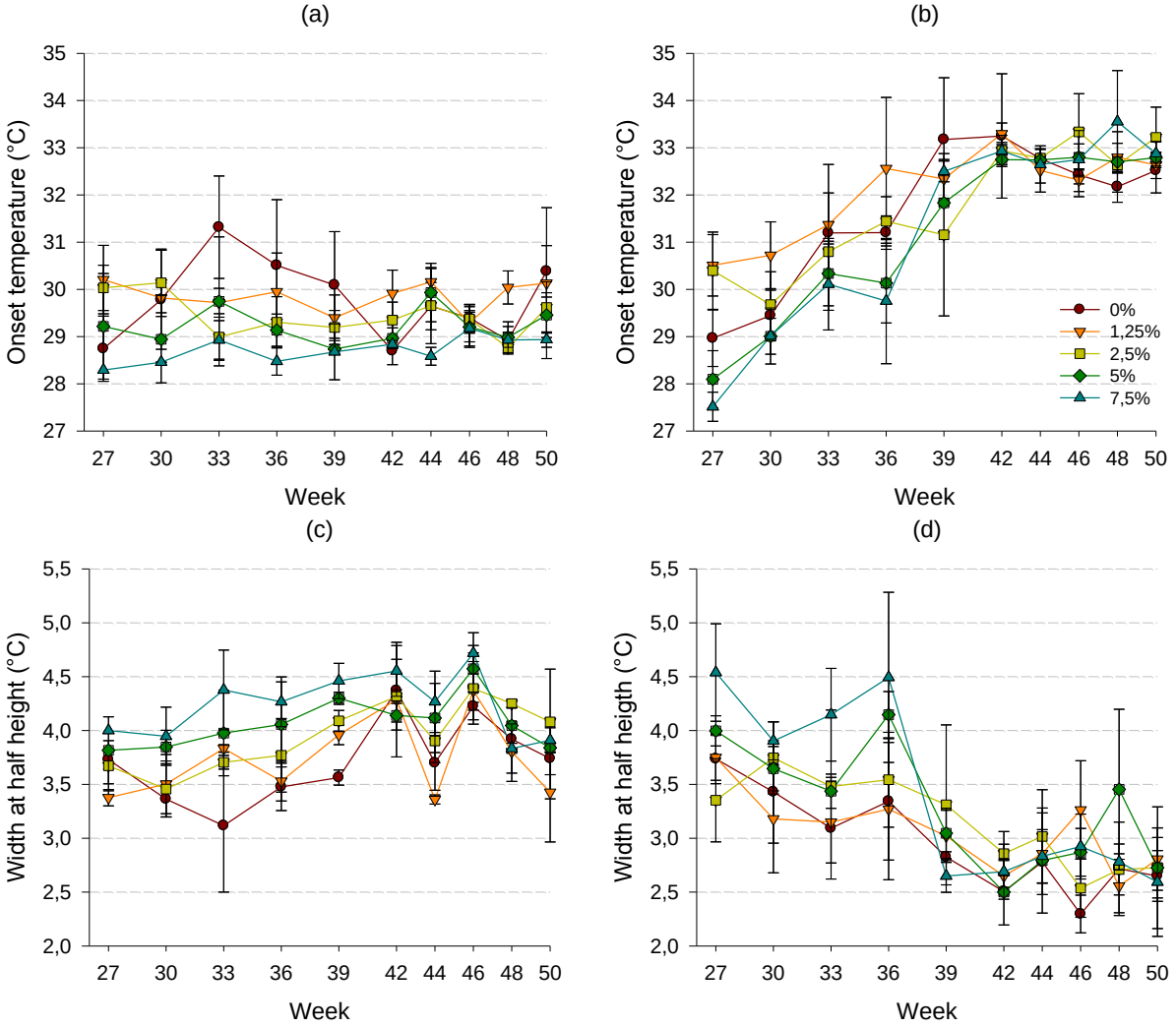


Figure 6.12 Onset temperature and width at half height as function of sampling time for the control chocolate (a,c) and chocolates with filling (b,d)

In summary: the melting behaviour of the filled chocolates with different DAG concentrations, as function of storage time was similar. The melting peak shifted to higher temperatures and became more narrow as function of storage time, while in the control samples, the peak became somewhat broader, with a slightly increased peak maximum. As a peak maximum of ~36°C is characteristic for β_{VI} crystals (Timms, 2003) the observed colour changes and the

dulling of the chocolate surface (dull, grey surface) can be ascribed to formation of fat bloom. The DAG present in the chocolate didn't prevent the formation of these fat bloom crystals.

6.4.2.4 Scanning electron microscopy

To confirm the previous finding, the surface of the chocolate samples was visualized by using scanning electron microscopy after 42 weeks of storage. On the right image of Figure 6.13b needle shape fat bloom crystals were observed at the surface of the chocolate in contact with the filing, while on the image of the corresponding chocolate without filling, no crystal formation was observed. In agreement with Timms (2003) fat bloom can be described as the development of a new phase in the chocolate fat, where the new phase becomes visible at the surface, often as clusters of large, frequently whitish crystals on the surface.

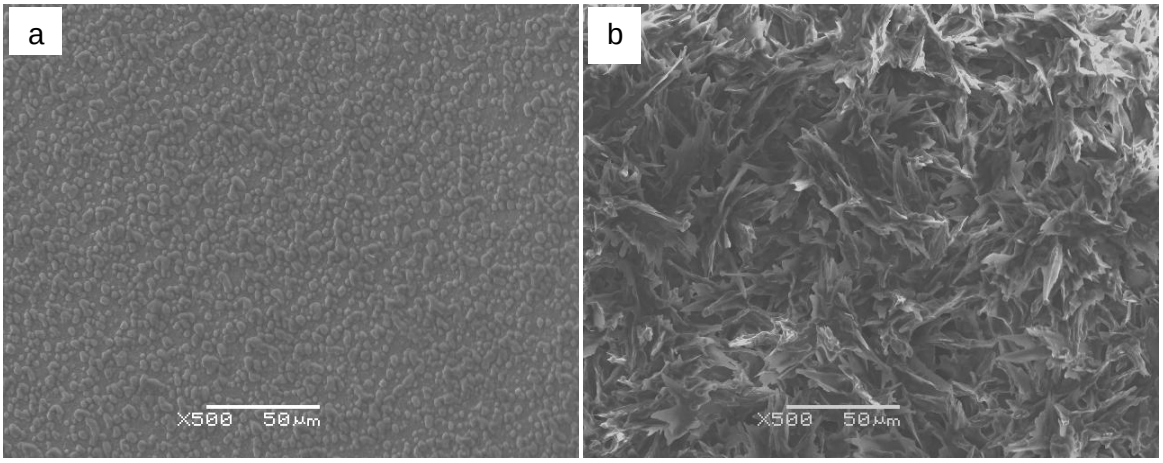


Figure 6.13 SEM image of chocolate 1.25% DAG (a) without filling (b) with filling (week 42)

6.4.2.5 Monitoring oil migration through the different layers

From the previous sections it could be concluded that the formation of fat bloom was correlated with the presence of a filling in contact with the chocolate. When a product contains two or more lipid containing components adjacent to each other fat migration will cause undesirable changes in confectionery products. The migration occurs when the lipids in the different components begin to interchange (Couzens and Wille, 1997). In this set up a typical hazelnut filling was used which mainly contained LOO, OOO, POO, predominantly liquid at room temperature. Triolein is the most predominant TAG followed by LOO and POO. Figure 6.14 compares the TAG composition of the chocolate with that of the filling. There was an apparent TAG concentration difference between the chocolate fat, containing mainly the typical CB TAG POP, POSt and StOSt and the filling fat. As the driving force for diffusion has been attributed to a gradient in TAG concentration, migration was likely to occur in the chocolate-hazelnut filling system. This suggests that while TAG migrated from

the filling to the chocolate, there is a corresponding migration of CB TAG into the filling (Smith et al., 2007a). The filling composition was not analyzed in this research.

To monitor the migration, the chocolate on top of the filling was cut in eight different layers of ± 50 μm. Only the even numbered layers were taken into consideration. The fat was extracted prior to HPLC analysis. Figure 6.15 unambiguously demonstrates that the TAG of the filling, LOO, OOO and POO, migrate to the chocolate as function of storage time. For that reason these TAG were used as markers for the migration through the chocolate. In order to avoid measurement variations due to sampling, the results are expressed as the ratio of these TAG to the amount of StOSt in the samples (Depypere et al., 2009b; Rousseau, 2006), so the dimensionless fractions [OOO/StOSt], [LOO/StOSt] and [POO/StOSt] were obtained in this research.

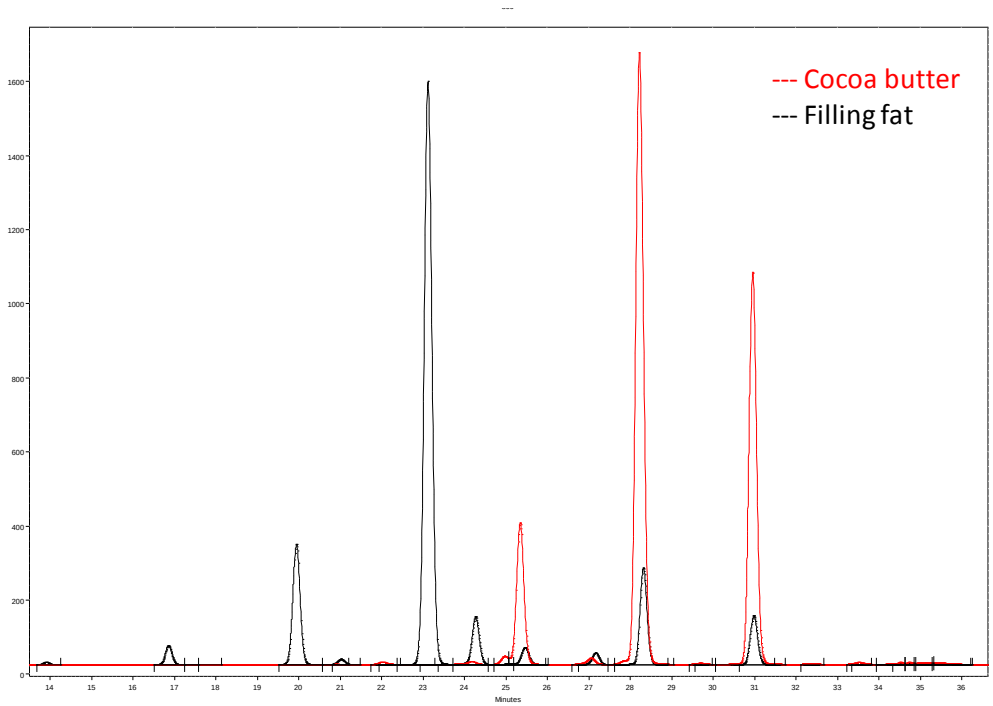


Figure 6.14 Overlay of the TAG profile of the hazelnut based filling and the fat from the chocolate (red chromatogram)

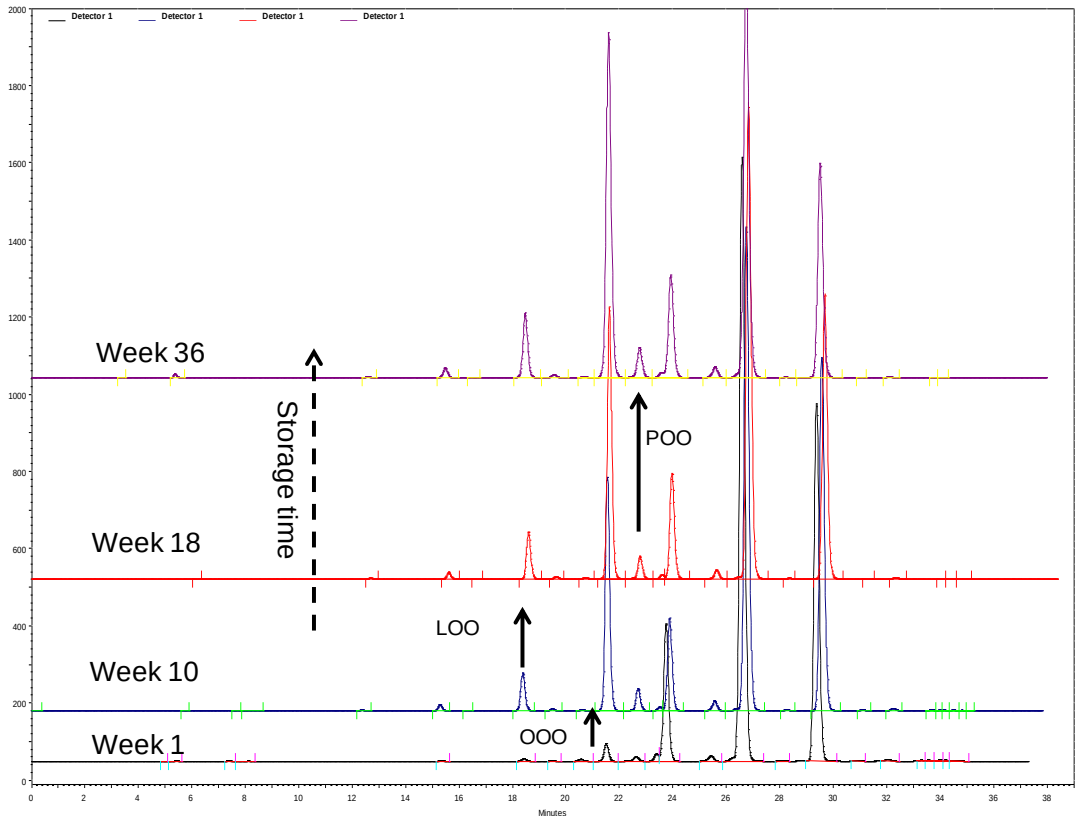


Figure 6.15 Overlay of HPLC chromatograms of layer 2 of chocolate with 2.5% DAG as function of storage time

The evolution of the ratios [LOO/StOSt] [OOO/StOSt] [POO/StOSt] in layer 2 is illustrated in Figure 6.16. The analyses showed a degree of scatter in the data; although overall trends may still be seen. No significant differences were detected between chocolates with different amounts of DAG. The filling specific TAG steadily increased in the chocolate as function of time. As OOO was the major TAG in the filling, it was also the one with the highest concentration in the chocolate. Figure 6.16d shows the ratio of the sum of the migrating TAG. The ratio increased almost linearly as function of time.

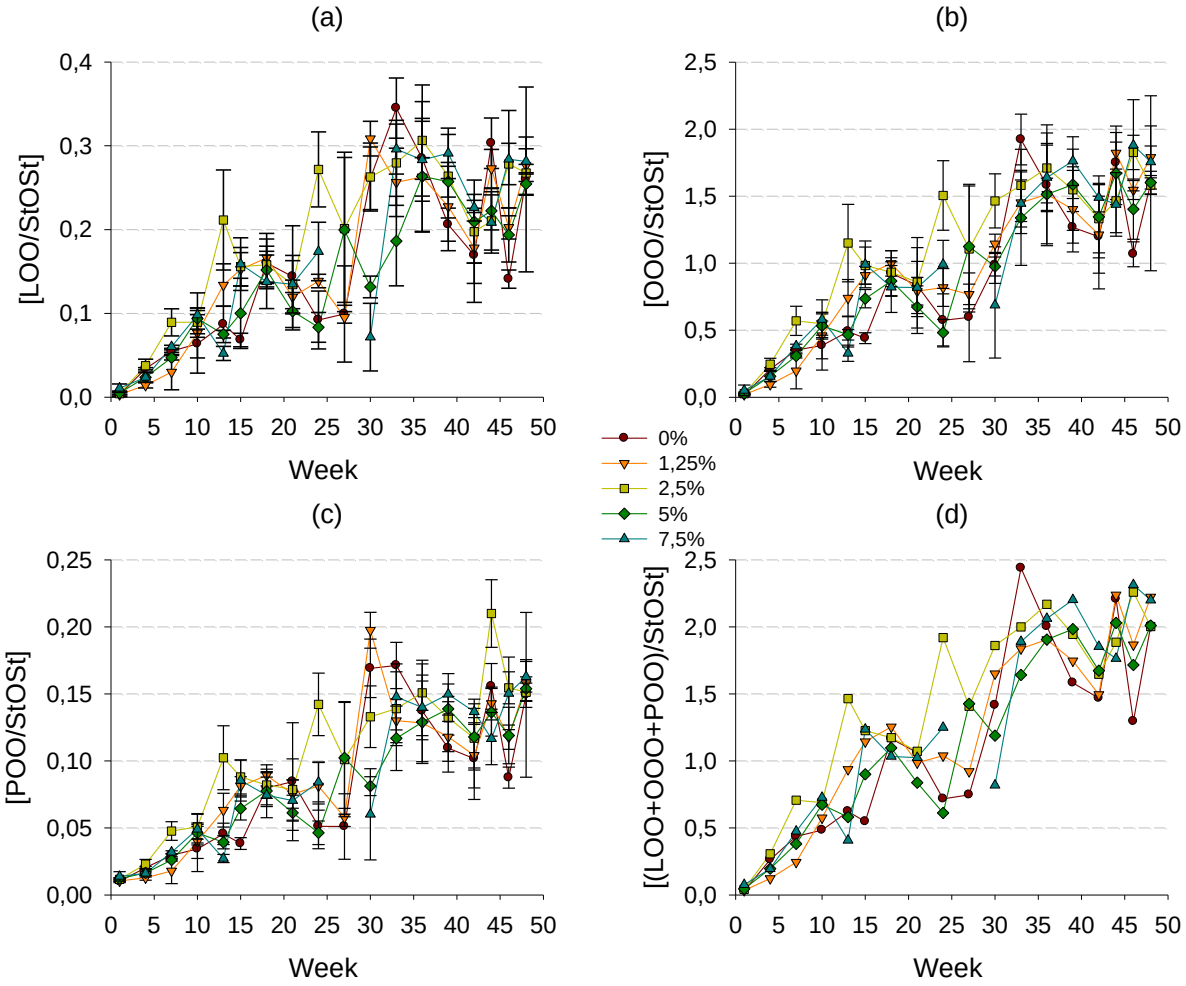


Figure 6.16 (a) [LOO/StOSt], (b) [OOO/StOSt] (c) [POO/StOSt] and (d) [(LOO+OOO+POO)/StOSt] in layer 2 as function of storage time for the chocolates with the different DAG concentrations

Molecular or Fickian diffusion is widely used by food engineers as a general model for mass transfer. To model fat migration, simplified solutions to Fick's second law of diffusion have been used (Galdamez et al., 2009). In order to describe the fat migration within filled chocolate products, Ziegleder et al. (1996a, b) proposed following equation:

$$\frac{\mathbf{m_t}}{\mathbf{m_s}} = \frac{\mathbf{A}\sqrt{\mathbf{D}\times\mathbf{t}}}{\mathbf{V}} = \frac{\sqrt{\mathbf{D}\times\mathbf{t}}}{\mathbf{d}} \qquad \text{Eq. 6-1}$$

where m_t is the mass concentration of fat migrating within the chocolate by time t (s), m_s is the mass concentration of fat migrating within chocolate at saturation point, V is volume (cm^3), A is the contact surface between phases (cm^2), d is the thickness of the chocolate layer (cm) and D is the coefficient of migration (cm^2s^{-1}). This has been effectively used by others to describe migration of liquid fat in chocolate (Aguilera et al., 2004; Altimiras et al., 2007; Khan and Rousseau, 2006; Miquel et al., 2001). The migration of OOO at 20°C in the

different layers for the chocolates with 0% and 5% DAG are presented in Figure 6.17. Within the different layers no differences were observed with or without the presence of extra CB DAG, indicating that the DAG didn't influence the migration process. A second observation is that migration was more or less linear but did not reach saturation within the end of the experiment. In a similar experiment, Ziegleder et al. (1996a) observed a linear increase at 20°C, slowing down at the end of the storage period. To verify this finding, the Eq. 6-1 was fitted to the data of layer 2. In the equation of the type $y = ax^b$, the value of b was fixed to 0.5 and fitted to the data of Figure 6.18. The curves of the model were deviating from the data points (0% DAG: R^2= 0.67; 5% DAG: R^2= 0.81). It can be seen that the model gave an overestimation of the amount of [OOO/StOSt] migrating at the beginning of the storage period. Typical for the model proposed by Ziegleder (1997) is that during the early stages of migration, the concentration of fat migrating into the chocolate increases linearly as function of the square root of time. This means that relatively large amounts are migrating during the first period and that this process will slow down at a later stage (Ziegleder, 1997). Similar to what was observed by Choi et al. (2007), the model based on Fickian diffusion with a constant diffusivity failed to accurately describe the migration of oil into the chocolate. A major concern while using the diffusion models is their lack of sensitivity towards the microstructural aspects of the chocolate layer. This means that besides diffusion, other factors like swelling and interaction between the oil and the cocoa butter can play a major role (Galdamez et al., 2009).

Gosh et al. (2002) and Khan and Rousseau (2006) describe an approach based on polymeric membrane solvent systems to study the impact of liquid fat migration in filled confections. Results can be analyzed using an empirical relationship of the type:

$$\frac{m_t}{m_\infty} = Kt^n \qquad \text{Eq. 6-2}$$

Where m_t and m_∞ are the mass diffused at time t and infinite time respectively. K is related to the polymer-solvent interaction and n describes the transport mechanism. If this exponent is 0.5, the diffusion is Fickian. Non-Fickian diffusion is observed for $0.5<n<1.0$. This anomalous behaviour is seen when phase changes occur during mass transfer, for example when there are components in the system that change from an amorphous to a crystalline state. Based on this approach, the n value was determined for the data in Figure 6.18. For both chocolates (0% DAG: R^2= 0.74, 5% DAG :R^2= 0.90) the n-values were in the same order of magnitude, i.e. 0.86 and 0.84, respectively, indicating non-Fickian behaviour. As migration progresses and the level of liquid oil in the chocolate increases, the physical characteristics

of the chocolate will change. In this case the third assumption of the Fickian model for transport is no longer valid, which states that the material does not physically change due to the expose to the penetrant (Khan and Rousseau, 2006). Additionally, another assumption, stating constant diffusivity was also not fulfilled. So as stated by Galdamez et al. (2009) the simplified models failed to accurately reproduce experimental data because the simplifying assumptions were not fulfilled.

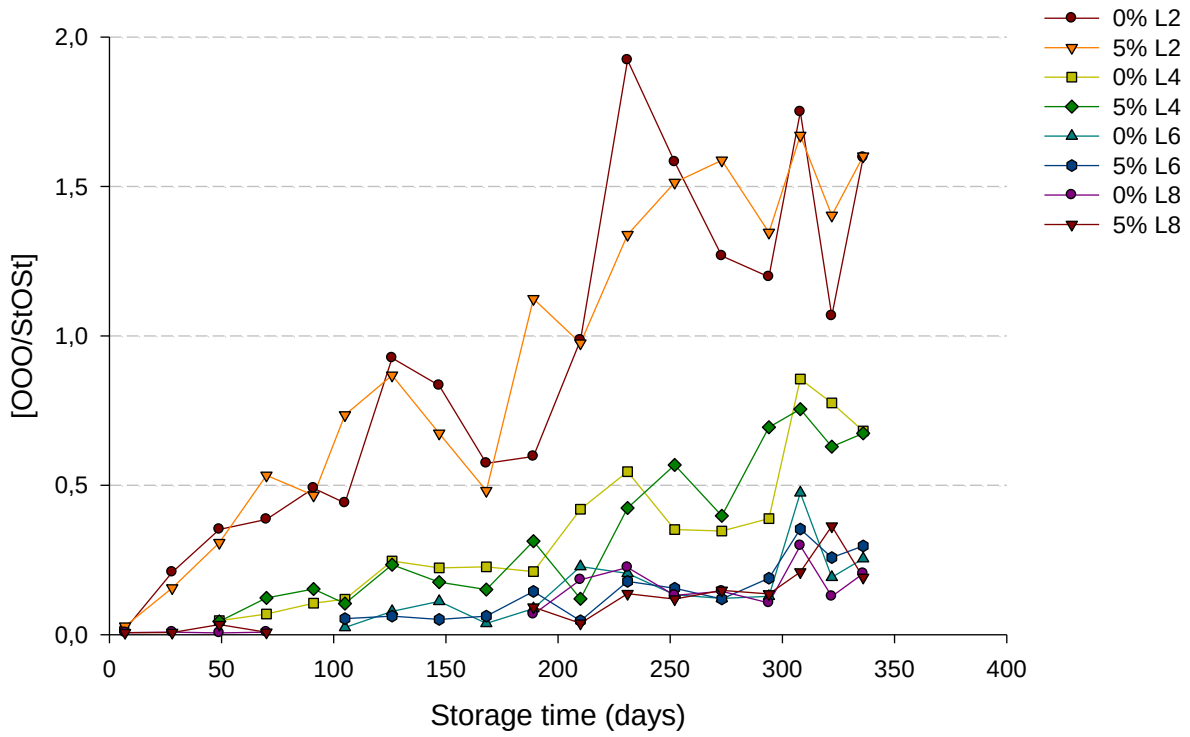


Figure 6.17 [OOO/StOSt] migration in the different layers for the control chocolate and the chocolate with 5% DAG.

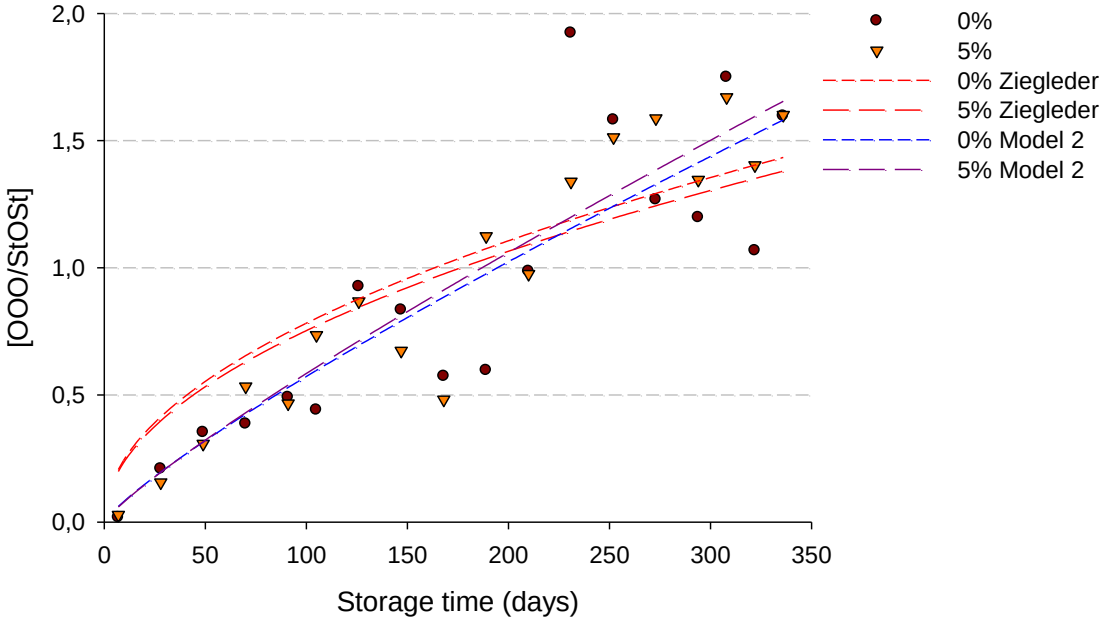


Figure 6.18 [OOO/StOSt] migration as function of storage time for chocolate 0% and 5% DAG with model fit of Ziegleder and Model 2

From Figure 6.17 it is also clear that migration is different within the different layers. The [OOO/StOSt] gradually decreased when from the interface with the filling to the surface of the chocolate. The ratio [OOO/StOSt] in layer 4 was about half the amount of layer 2. Layer 6 and 8 contained both about half the amount of layer 4, so the ratio [OOO/StOSt] was very similar in these two layers. To visualize how this gradient evolves as function of storage time, the [OOO/StOSt] was plotted as function of chocolate thickness in Figure 6.19. The migration process didn't progress proportionally through the different layers as no linear gradient was observed. Similar to the findings of Marty et al. (2005) and Smith et al. (2007a) in model systems of oil-cocoa butter, the overall trend was that the concentration of the filling fat was the highest near the interface with the filling, dropping off with distance from the interface.

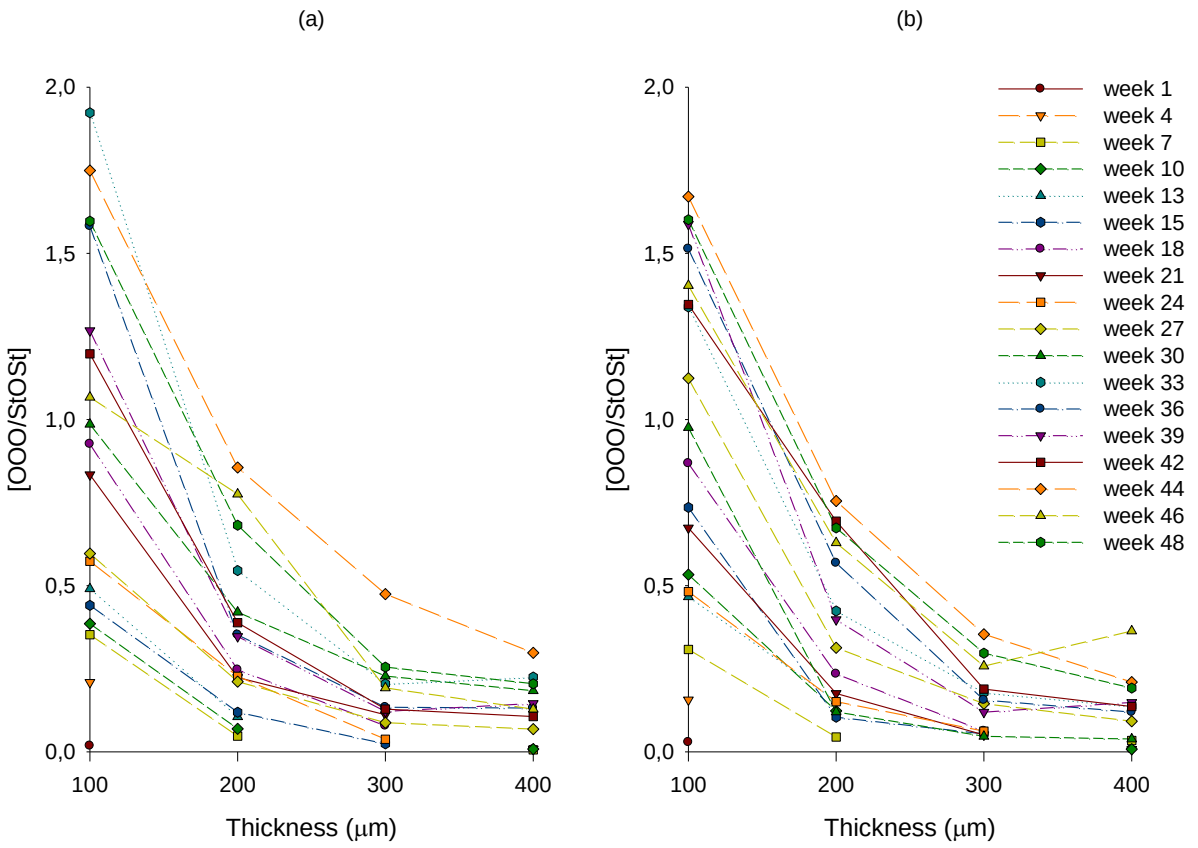


Figure 6.19 [OOO/StOSt] gradient as function of the chocolate thickness for the whole storage period (a) chocolate 0% (b) chocolate 5% DAG

6.4.2.6 Melting profile of the different chocolate layers

The melting behaviour of the different layers was also monitored as function of time. Firstly, the effect within one layer, layer 8, will be discussed for the chocolate with 0% and 5% DAG (Figure 6.20). Secondly, the difference between the layers of a chocolate are demonstrated in Figure 6.21. Chocolate 5% was again used as a representative sample. Unless mentioned, the presented results are representative for the other chocolates with DAG.

– *Effect of oil migration in layer 8 (Figure 6.20)*

In comparison to the discussion of section 6.4.2.3, the melting properties of the bulk, and thus not of the surface of layer 8, are described here.

The melting enthalpy of chocolate 5% had the tendency to be lower than that of the chocolate with 100% cocoa butter but as function of time their peak area remained constant. For the other parameters, no significant differences were observed as function of the DAG concentration. The melting peak shifted to a higher temperature of ~36°C, so a polymorphic transformation to β_{VI} occurred during storage. At the end of the storage, the onset temperature increased with 1°C. The width of the peak showed values between 5 and 6°C

during the whole storage period. Compared to the control sample without filling (Figure 6.11 and Figure 6.12) the melting peak has become broader and has shifted to higher temperatures.

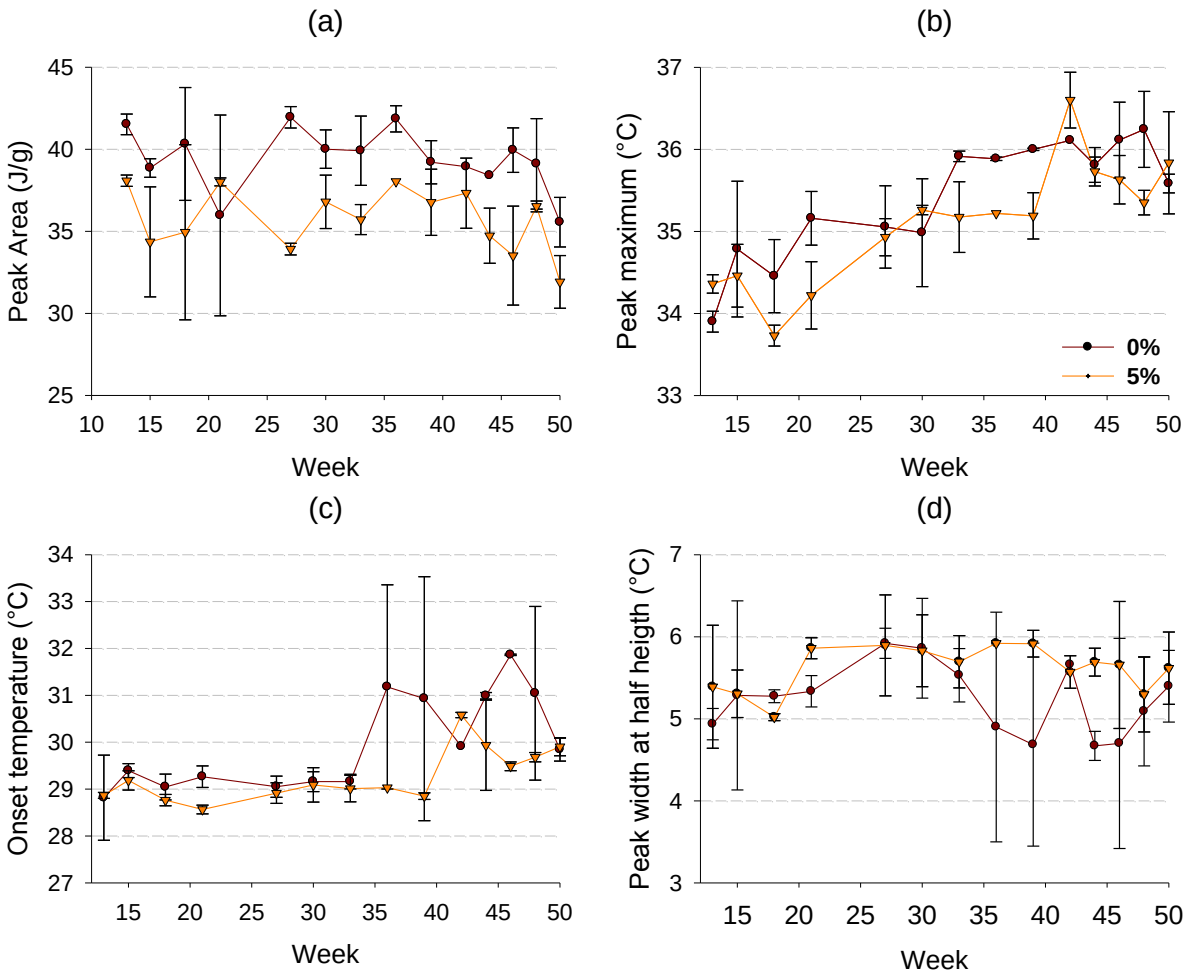


Figure 6.20 Peak area (a), peak maximum (b), onset temperature (c) and width at half height (d) for layer 8 as function of sampling time for the chocolate with 0 (●) and 5% DAG (▼)

– *Effect of oil migration through the different layers (Figure 6.21)*

The HPLC results have shown that more filling TAG were present in layer 2. This was reflected in a lower peak area and peak maximum. The peak area of layer 4 also showed the tendency to be lower as more oil was present compared to the upper layers. The peak maximum of this layer varied between 35 – 36°C while for layer 6 and 8 an increase was observed during storage. This confirmed the finding of Smith et al. (2007a) that the chocolate the closest to the filling experienced both greater degree of migration and faster transformation. The onset temperature shifted to temperature around 30°C after 30 weeks storage while the peak width didn't significantly changed, showing values between 5 – 6°C.

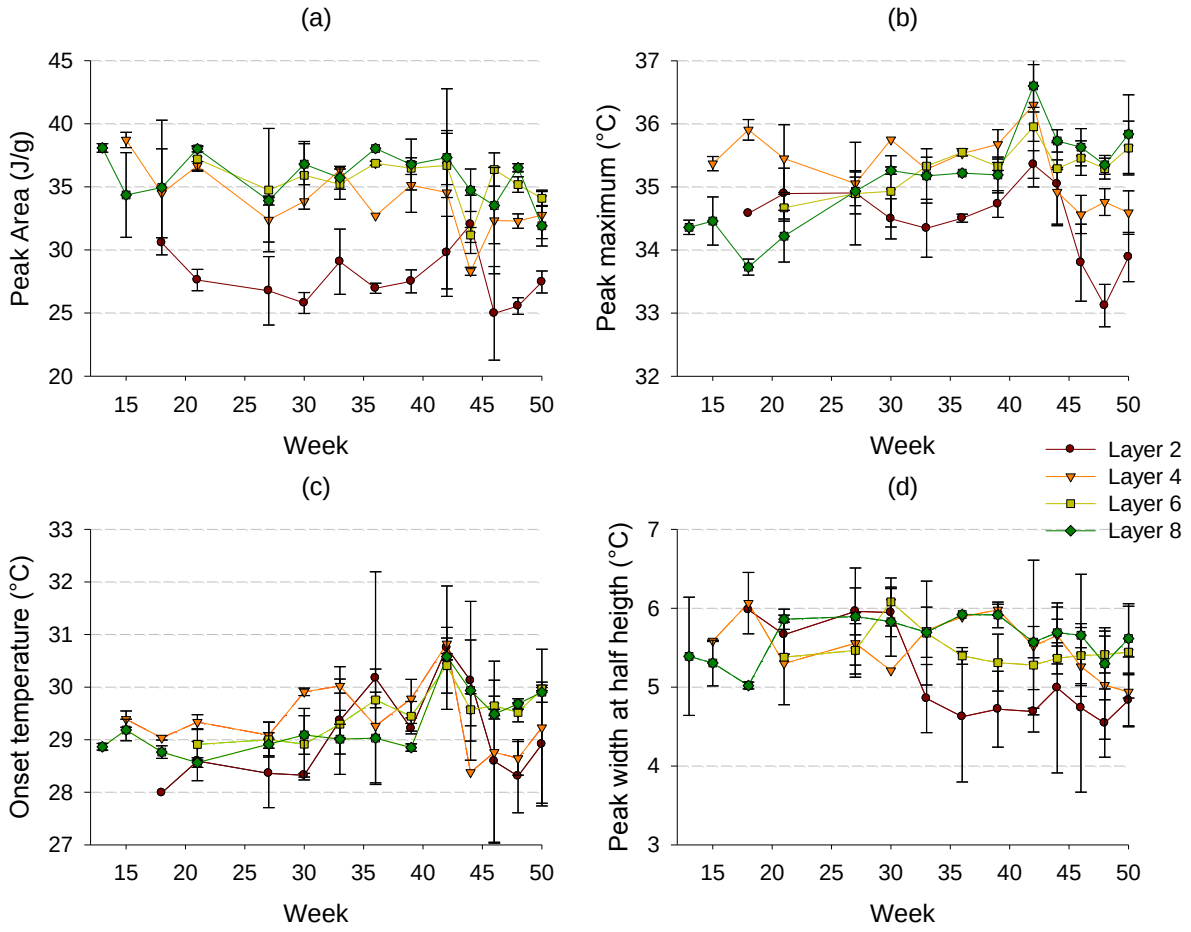


Figure 6.21 Peak area (a), peak maximum (b), onset temperature (c) and width at half height (d) as function of sampling time for the chocolate with 5% DAG.

6.4.2.7 Migration and the occurrence of fat bloom

When a chocolate is in contact with a filling, the two fat phases will interact and an exchange of TAG will occur. This oil migration process was responsible for a colour change and a duller chocolate surface as this was not observed for the control chocolates without filling. So the liquid oil is migrating through the chocolate, thereby dissolving CB TAG. As mixing takes place, the equilibrium between the solid and liquid phases is disturbed and some of the previously solid material may dissolve in the new liquid phase. Thus the TAG that are generally considered to be solid at a certain temperature are able to migrate due to dissolution in the liquid phase, movement to the adjacent layer and when they reach the surface, the TAG re-crystallize, forming fat bloom (Smith et al., 2007a; Ziegler et al., 2004). The different analyses have shown that between 30 – 33 weeks of storage a change occurred at the surface of the chocolate. This was observed for the chocolates with and without CB DAG. It seemed that around that time, as defined by Timms (2003), a new phase of larger crystals developed at the chocolate surface. It was confirmed by SEM that crystals

protruded at the chocolate surface. Moreover, the mean pixel values in the image analysis procedure showed an increased value and the melting characteristics of the chocolate samples at the surface changed. The melting enthalpy doubled, onset and peak maximum shifted to higher temperatures and the peak width became narrower. The findings indicated that re-crystallization occurred and that β_{VI} crystals were formed. Linking these findings with the results of the HPLC analysis, i.e., oil migration, for all the samples, the corresponding [OOO/StOSt] was still quite low at the surface but it was sufficient to trigger the polymorphic transformation. This observation supported the finding of Smith et al. (2007a) that small additions of nut oil can have an impact on the rate of transformation. The migration-induced fat bloom was probably the result of Ostwald ripening as described by Ziegler et al. (2004). The smaller crystals have the ability to dissolve in the liquid oil so that the smaller crystals melt and the larger ones grow. Smaller crystals can also fuse to form larger ones. DSC analyses of the different layers also confirmed that this re-crystallization process was a bulk phenomenon.

As the DAG chocolates showed similar results compared to the reference chocolate, it can be assumed that within the setup of this study, they didn't play a significant role nor in preventing nor in promoting the studied processes. The CB DAG may have changed the microstructure but not in a way that oil migration was delayed. They may have played a role in the liquid-solid re-crystallization behaviour but not in a way that re-crystallization was prevented. It's possible that the CB DAG were too much alike CB TAG to induce significant changes.

6.5 Conclusion

In the first part of this study, 25% (on total fat base) of the cocoa butter in the chocolate was replaced by CB DAG. Up to 5% DAG, the physical characteristics of the chocolates were not significantly influenced compared to the reference chocolate. Higher amounts of DAG slightly decreased the peak maximum and the Casson yield stress was significantly higher. This can be an interesting finding as chocolate flow properties can be adjusted by adding a CB based emulsifier. A fractionation of the CB DAG may again be of interest as it was seen that mainly the high-melting DAG influenced the chocolate properties.

In the second part of the study, the performance of the DAG chocolates containing up to 7.5% DAG on fat base, on the occurrence of fat bloom was evaluated. Model systems with and without filling were used for this purpose. It was seen that the quality characteristics of the plain chocolates slightly changed during the one year storage period. In the composed chocolate products, DSC and SEM demonstrated that the oil migration was a triggering

factor for the re-crystallization to β_{VI} crystals, protruding through the chocolate surface protruding through the chocolate surface resulting in a greyer and duller appearance. The model based on Fickian diffusion with a constant diffusivity failed to accurately describe the migration of oil into chocolate. Non-Fickian behaviour occurs as besides migration, interaction between oil and cocoa butter can also play a major role. The present DAG did not delay oil migration, nor prevented the appearance of migration fat bloom.

General conclusions

Cocoa butter forms the continuous phase of chocolate. The crystallization of cocoa butter provides unique characteristics of texture, mouth feel, flavour release to chocolate. Because of its relatively simple composition, more than 75% exhibit a symmetrical conformation with oleic acid at the *sn*-2 position, it's highly polymorphic as at most six polymorphic forms have been observed. The chemical composition has a major impact on the crystallization kinetics of a cocoa butter. This will affect the formation of the crystal network, which in turn determines many of the macroscopic properties.

In this research two tools were used to change the chemical composition and thus the functionality of the cocoa butter and thus consequently modify the functionality of chocolate.
In a first part of this research, three crude cocoa butters with or without silica pretreatment were subjected to a steam refining in a packed column at five temperatures. The silica treatment and/or packed column steam refining significantly changed the physicochemical properties of the treated cocoa butters. The major effect of the silica pretreatment was the complete removal of phosphorous (thus phospholipids), iron and alkaline components. An interesting finding was that the removal of the pro-oxidant iron doubled the oxidative stability. Compared to a traditional bleaching process, not all the alkaloids, like theobromine and caffeine, were removed. The removal of the alkaloids during this steam refining can cause problems as these deposit on the vapour scrubbers, decreasing its efficiency. The different refining conditions also resulted in cocoa butters with different colours, mainly interesting for their application in white chocolate. The main effect of the steam-refining step was the removal of the FFA at increased temperature ($T \geq 200°C$). The changes in the physical properties of cocoa butter were mainly related to the removal of these fatty acids.

The impact of the applied refining conditions on milk chocolate quality were verified. It was demonstrated that the refining of cocoa butter influenced the flow behaviour of the milk chocolate. It was observed that an increased packed column temperature, coinciding with the removal of FFA, resulted in a lower yield stress but a higher viscosity. FFA presumably played a role at the interface between the solid particles and the continuous fat phase but the exact mechanism remained unclear. The possibility to adjust the flow properties of molten chocolate is important for chocolate manufacturers, to optimize their processes and formulations and to reduce the costs. Therefore, it could be interesting to further explore the

influence of free fatty acids on flow behaviour of chocolate. The main conclusion of this study is that by tuning the refining conditions (pretreatment or no pretreatment, temperature of the refining process), the cocoa butter properties can be directed to suit the desired chocolate properties.

In the second part of this research, the composition of cocoa butter was more drastically changed. As diacylglycerols have distinct physicochemical and nutritional properties compared to triacylglycerols, the objective was to synthesize cocoa butter based diacylglycerols.

An enzyme catalyzed glycerolysis reaction was the method of choice as it's a convenient and fast method, without the use of solvents. After optimizing the different reaction parameters, the reaction yielded around 50% diacylglycerols. Short path distillation was then used to obtain highly purified cocoa butter diacylglycerols.

A next step was to determine the physicochemical properties of the produced cocoa butter diacylglycerols. They contained around 50% monounsaturated, 37% disaturated and 11% diunsaturated diacylglycerols. Co-crystallization and intersolubility resulted in three main melting areas. The diacylglycerols were blended with cocoa butter to screen their possible applicability in confectionary products. In the isosolid phase diagram a eutectic minimum indicated the diacylglycerols had a softening effect. The crystallization enthalpies also revealed that interactions between the acylglycerols occurred. Compared to cocoa butter, the crystallization enthalpy of the diacylglycerols almost doubled. This may have implications on for example heat transfer in crystallizers or scraped surface heat exchangers but this can also be important for product functionality as they may exert a natural cooling sensation.

A multi-methodological approach was used to study the isothermal crystallization of cocoa butter in the presence of maximum 10% cocoa butter diacylglycerols over a period of four hours. At an isothermal crystallization temperature of 20°C, cocoa butter crystallizes in a two-step process: the α crystals formed during the first step, transform to β' in the second step. It was observed that diacylglycerols influenced nucleation and polymorphic transition. The high-melting disaturated diacylglycerols changed the saturation conditions of the melt and acted as seed crystals. The induction time of the first crystallization step was therefore inversely related to the amount of diacylglycerols. The subsequent polymorphic transition on the other hand, was delayed by the presence of the diacylglycerols as they seem to stabilize the hexagonal packing. The good match in fatty acid composition between the diacylglycerols and cocoa butter may have contributed to this effect. Up to 2.5%

diacylglycerols, besides solid-solid transition, β' crystals were also directly formed from the melt. At higher concentrations, the β' formation was only α-mediated. It was observed that when 5% or more CB DAG were present, a β diffraction peak appeared at the end of the time interval of the analysis. This may indicate that the subsequent β' to β polymorphic transition may be induced by the presence of the diacylglycerols. Further research is necessary to further explore this observation.

Finally, in the last chapter, the diacylglycerols were applied in a dark chocolate. Up to 5% diacylglycerols the physical characteristics of the chocolates were not significantly influenced. Higher amounts of DAG slightly decreased the peak maximum and the Casson yield stress was significantly higher. This may be of practical interest as this implies that the chocolate flow properties can be adjusted by adding a CB based emulsifier.
As migration fat bloom remains one of the major quality issues in composed chocolate products, it was investigated whether cocoa butter based diacylglycerols could delay or prevent this phenomenon. Model systems were used for this purpose and different techniques were used to monitor oil migration and fat bloom formation. The changes in the triacylglycerol profile through the different chocolate layers as function of time demonstrated that non-Fickian diffusion occurred. Besides diffusion, microstructural factors of chocolate and phase behaviour of the present lipids will also play a role. It was observed that the quality characteristics of the plain chocolates only slightly changed during the one-year storage period. In the composed chocolate products, DSC and SEM demonstrated that the oil migration was an activating factor for the re-crystallization to β_{VI} crystals, protruding through the chocolate surface. Only a small amount of liquid oil was necessary to induce the re-crystallization. As the DAG chocolates showed similar results compared to the reference chocolate, it can be assumed that they did not play a significant role nor in preventing nor in promoting the studied processes within the setup of this study. The CB DAG may have changed the microstructure, but not in a way that oil migration was delayed. They may have played a role in the liquid-solid re-crystallization behaviour, but not in a way that re-crystallization was prevented.

The diacylglycerols showed interesting perspectives in changing the functionality of cocoa butter. However, the major disadvantage of the produced cocoa butter diacylglycerols was their high amount of residual solid fat above body temperature resulting in a waxy mouth feel and so limiting their applicability. A possible solution could be the fractionation of these diacylglycerols to remove the high-melting diacylglycerols. The olein fraction may offer the possibility to replace a part of the cocoa butter in a chocolate formulation, thereby combining

a healthier fatty acid profile (less saturated fatty acid) with the nutritional benefits of the diacylglycerols. However, the diacylglycerols considerably changed the polymorphic behaviour of the fat crystal network in a way that the typical macroscopic properties of a chocolate may be affected.

These results form an interesting scientific basis to further investigate the physicochemical properties and functionality of diacylglycerols.

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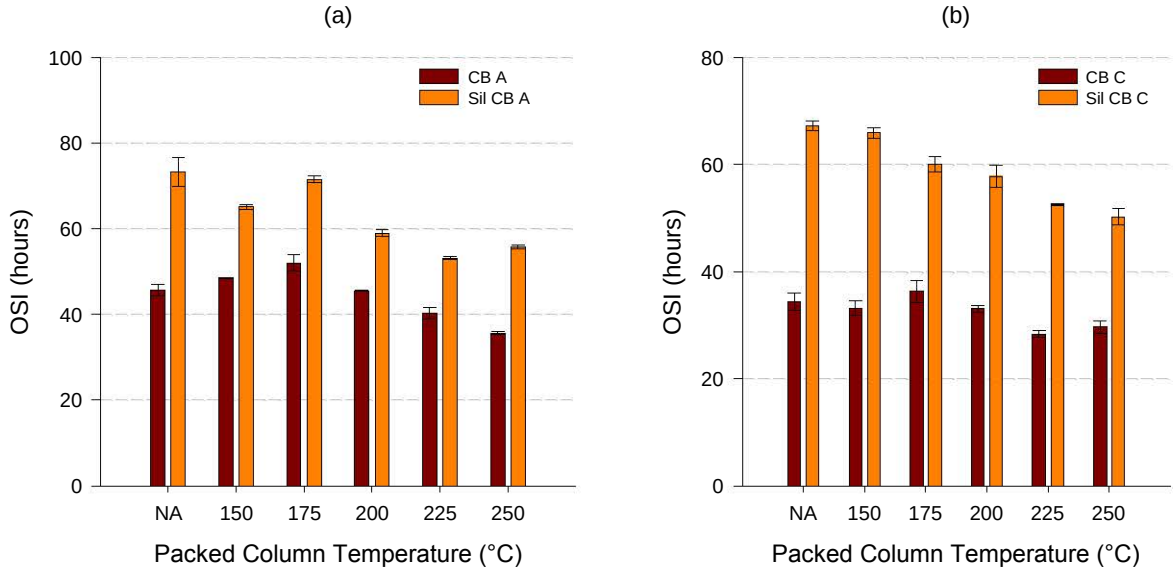
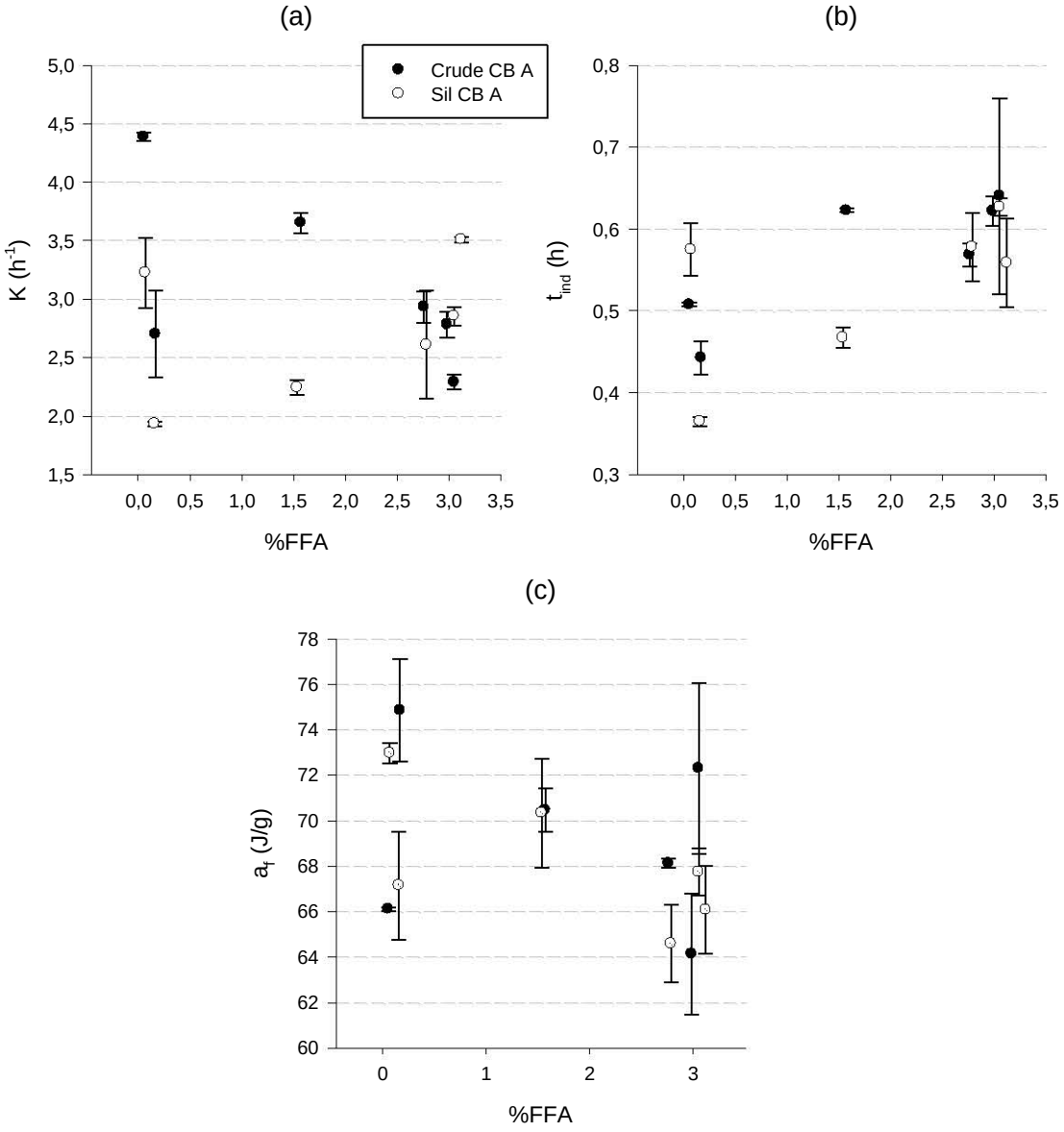
Annex I

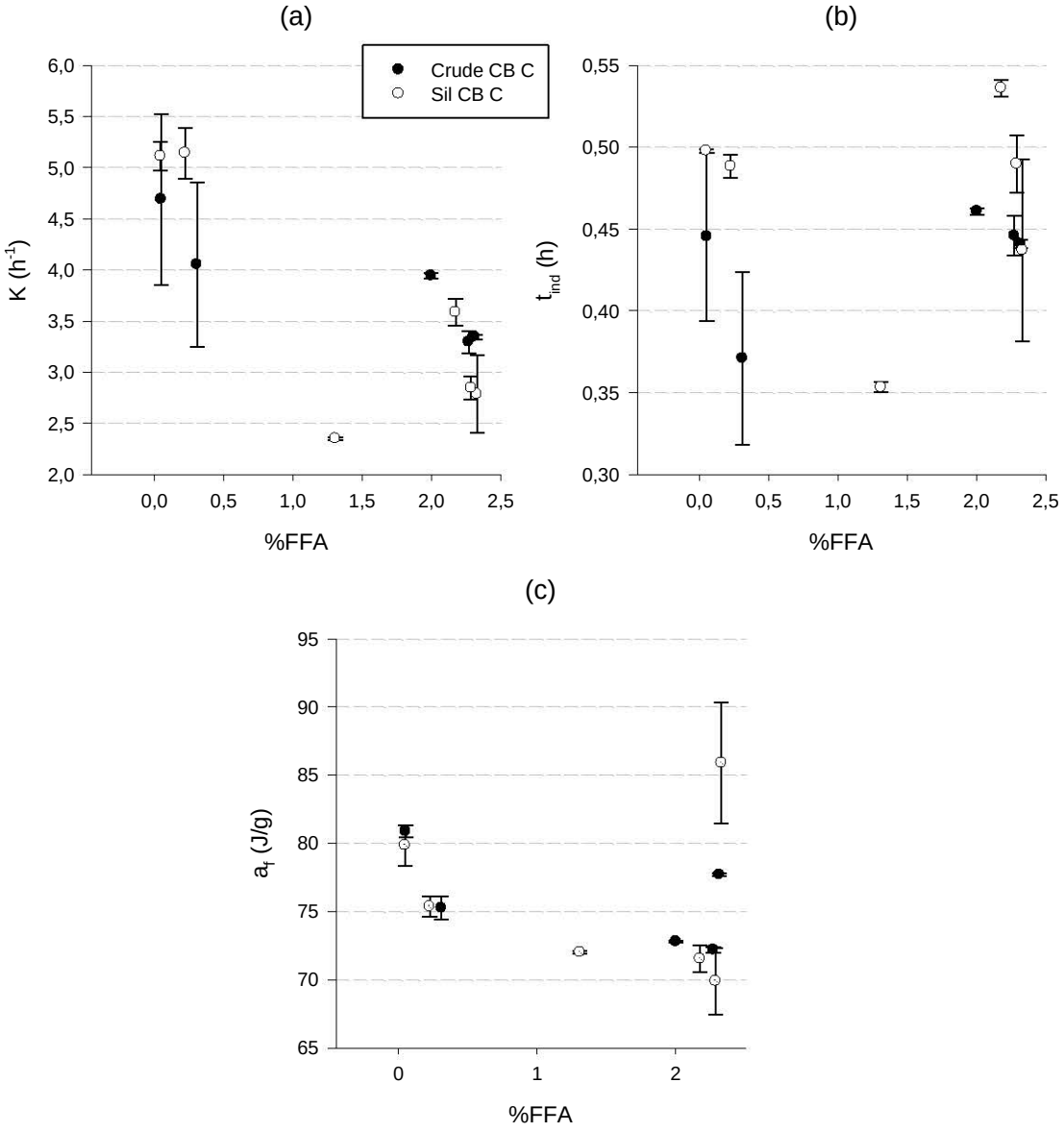
Annex I-A Oil stability index (OSI) as function of packed column temperature for crude and silica pretreated cocoa butter (a) CB A (B) CB C



Annex I-B Foubert-model parameters K (a), a (b) and t_{ind} (c) as function of the residual FFA for the refined samples of CB A



Annex I-C Foubert-model parameters K (a), a (b) and t_{ind} (c) as function of the residual FFA for the refined samples of CB C



Annex I-D Optimization of the milk chocolate production process

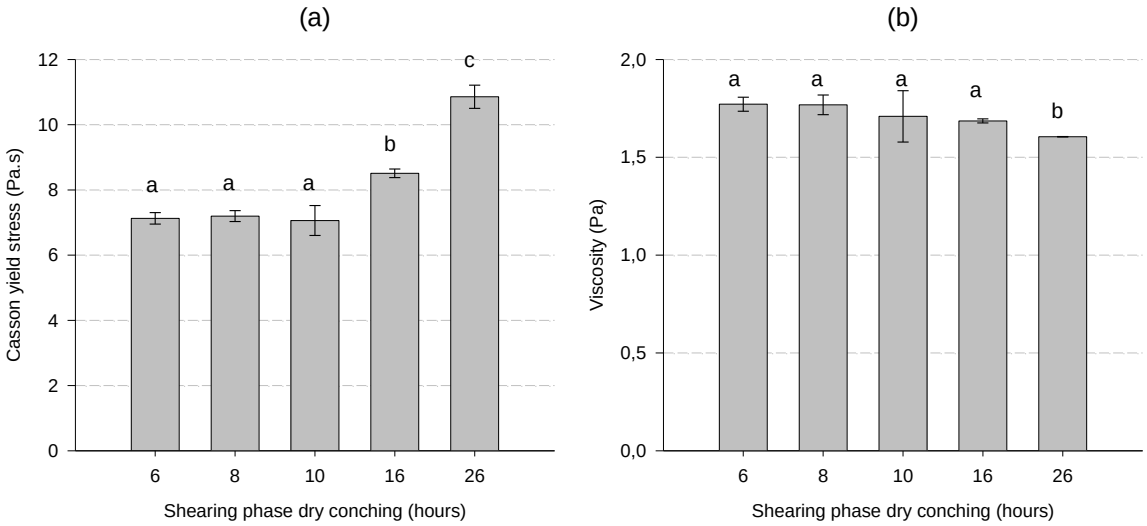


Figure I-B (a) Yield stress and (b) viscosity as function of dry conching length. Samples that are not significantly different are marked with the same letter

Conching length (h)	6	8	10	16	26
D[90] (µm)	28.4 ± 1.3	29.5 ± 1.3	28.8 ± 1.9	27.5 ± 3.3	25.4 ± 1.8
D[4,3] (µm)	11.9 ± 0.4	12.5 ± 0.6	12.5 ± 0.7	11.7 ± 1.4	10.9 ± 0.6

Annex II

Annex II-A Factor levels of a fractional Face-Centered Central Composite design with five factors and the corresponding response

NR	Reaction time (h)	Enzyme load (wt% oil)	React. Temp (°C)	Substr ratio (mol oil/glycerol)	Water content (wt% glycerol)	DAG (%)
1	3	3	45	0.25	6	17.85
2	15	3	45	0.25	0	21.74
3	3	15	45	0.25	0	34.6
4	15	15	45	0.25	6	36.62
5	3	3	75	0.25	0	36.17
6	15	3	75	0.25	6	36.74
7	3	15	75	0.25	6	45.04
8	15	15	75	0.25	0	37.53
9	3	3	45	2	0	16.02
10	15	3	45	2	6	31.66
11	3	15	45	2	6	34.17
12	15	15	45	2	0	49.67
13	3	3	75	2	6	32.09
14	15	3	75	2	0	46.58
15	3	15	75	2	0	46.07
16	15	15	75	2	6	47.01
17	9	9	60	1.13	3	43.45
18	9	9	60	1.13	3	44.76
19	9	9	60	1.13	3	46.01
20	9	9	60	1.13	3	45.3
21	9	9	60	1.13	3	44.38
22	9	9	60	1.13	3	51.73
23	9	9	60	1.13	3	44.66
24	9	9	60	1.13	3	44.51
25	3	9	60	1.13	3	40.56
26	15	9	60	1.13	3	46.81
27	9	3	60	1.13	3	49.3
28	9	15	60	1.13	3	48.03
29	9	9	45	1.13	3	45.29
30	9	9	75	1.13	3	47.79
31	9	9	60	0.25	3	44.77
32	9	9	60	2	3	41.73
33	9	9	60	1.13	0	45.45
34	9	9	60	1.13	6	42.25
35	9	9	60	1.13	3	39.14
36	9	9	60	1.13	3	42.44

Annex II-B ANOVA results for quadratic model

Response 1 % DAG
Backward Elimination Regression with Alpha to Exit = 0.100
Forced Terms Intercept, Block 1

Removed	Coefficient Estimate	t for H_0 Coeff=0	Prob > |t|	R-Squared	MSE
AE	0.014	0.013	0.9898	0.8899	16.66
BE	-0.18	-0.18	0.8633	0.8897	15.65
C^2	-0.58	-0.23	0.8214	0.8893	14.78
CE	-0.23	-0.24	0.8107	0.8889	14.01
CD	-0.28	-0.30	0.7702	0.8884	13.33
B^2	1.41	0.62	0.5407	0.8861	12.93
BD	0.58	0.65	0.5261	0.8838	12.57
E	-0.58	-0.69	0.4968	0.8811	12.27
DE	-1.23	-1.40	0.1753	0.8705	12.78
E^2	-2.99	-1.41	0.1732	0.8594	13.30
AB	-1.48	-1.62	0.1182	0.8440	14.17

ANOVA for Response Surface Reduced Quadratic Model
Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Block	244.79	1	244.79			
Model	1915.93	9	212.88	15.03	< 0.0001	significant
A-Reaction time	*149.01*	*1*	*149.01*	*10.52*	*0.0033*	
B-Enzyme Load	*455.92*	*1*	*455.92*	*32.19*	*< 0.0001*	
C-Temperature	*424.38*	*1*	*424.38*	*29.96*	*< 0.0001*	
D-Substrate molar ratio	*64.00*	*1*	*64.00*	*4.52*	*0.0436*	
AC	*5 0.98*	*1*	*50.98*	*3.60*	*0.0694*	
AD	*141.61*	*1*	*141.61*	*10.00*	*0.0041*	
BC	*119.46*	*1*	*119.46*	*8.43*	*0.0076*	
A^2	*66.20*	*1*	*66.20*	*4.67*	*0.0404*	
D^2	*79.95*	*1*	*79.95*	*5.64*	*0.0255*	
Residual	354.13	25	14.17			
Lack of Fit	*301.96*	*17*	*17.76*	*2.72*	*0.0761*	*not significant*
Pure Error	*52.17*	*8*	*6.52*			
Cor Total	2514.86	35				

Std. Dev.	3.76	R-Squared	0.8440
Mean	40.78	Adj R-Squared	0.7878
C.V. %	9.23	Pred R-Squared	0.6852
PRESS	714.70	Adeq Precision	16.126

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High
Intercept	45.54	1	0.92	43.64	47.44
Block 1	-0.46	1			
Block 2	0.46				
A-Reaction time	2.88	1	0.89	1.05	4.70
B-Enzyme Load	5.03	1	0.89	3.21	6.86
C-Temperature	4.86	1	0.89	3.03	6.68
D-Substrate molar ratio	1.89	1	0.89	0.059	3.71
AC	-1.79	1	0.94	-3.72	0.15
AD	2.97	1	0.94	1.04	4.91
BC	-2.73	1	0.94	-4.67	-0.79
A^2	-4.39	1	2.03	-8.58	-0.21
D^2	-4.83	1	2.03	-9.02	-0.64

Final Equation in Terms of Coded Factors:

% DAG =+45.54+2.88*A+5.03*B+4.86*C+1.89*D-1.79*A*C+2.97*A*D-2.73*B*C-4.39*A^2-4.83*D^2

Final Equation in Terms of Actual Factors:

% DAG =-27.40781+3.22905 * Reaction time+2.66046 *Enzyme Load+0.77545 * Temperature+11.24635 *Substrate molar ratio-0.019833 *Reaction time * Temperature+0.56667 * Reaction time * Substrate molar ratio-0.030361 * Enzyme Load * Temperature-0.12206 * Reaction time^2-6.30730 * Substrate molar ratio^2

Annex III

Annex III TAG composition of cocoa butter

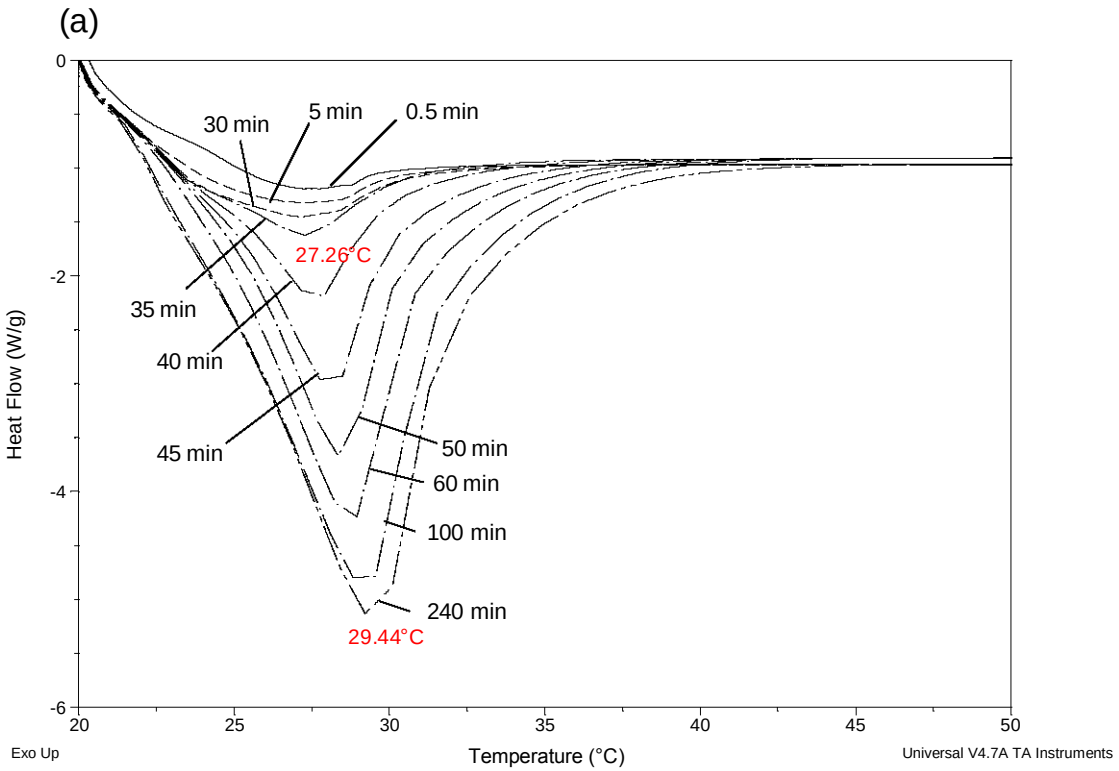
TAG*	%
LLO	0.07
PLL	0.16
MLP	0.06
OOL	0.40
POL	0.44
PLP	1.77
MPP	0.00
OOO	0.34
POO	2.82
POP	18.09
PPP	0.12
StOO	3.20
POSt	39.70
PPSt	0.48
StOSt	27.88
PStSt	0.64
StOA	2.96
N.I.	0.86

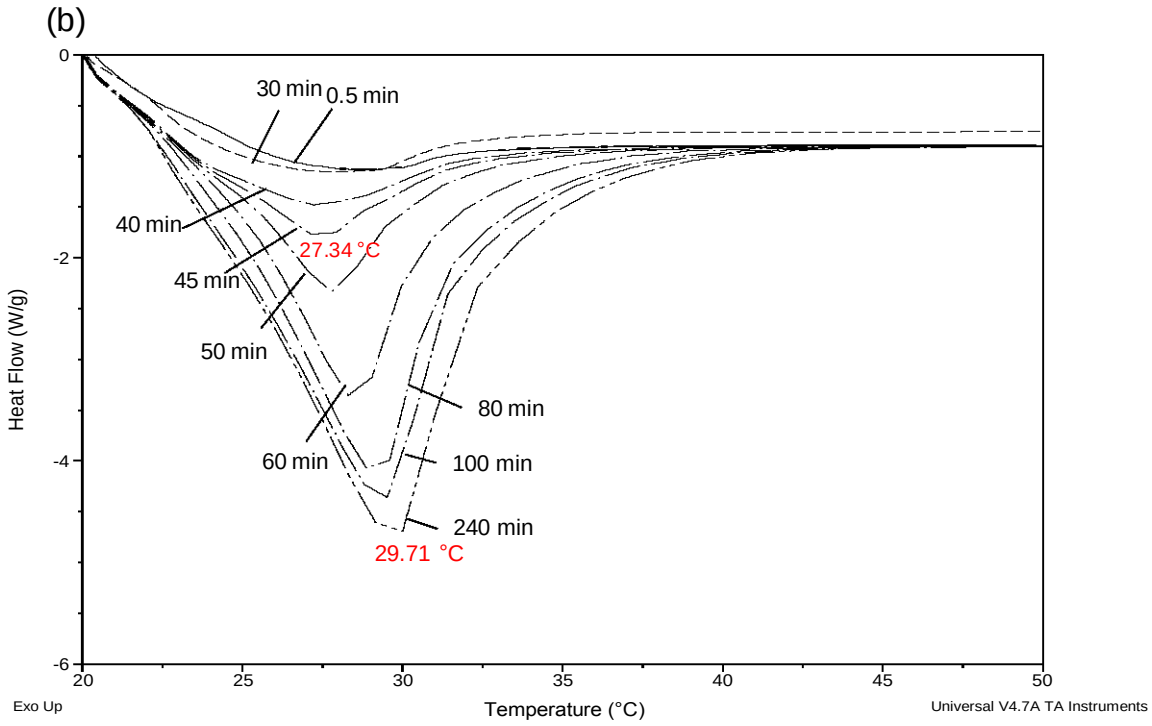
*M, myristic; P, palmitic; St, stearic; A, arachidonic; O, oleic; L, linoleic acid

N.I. Not identified

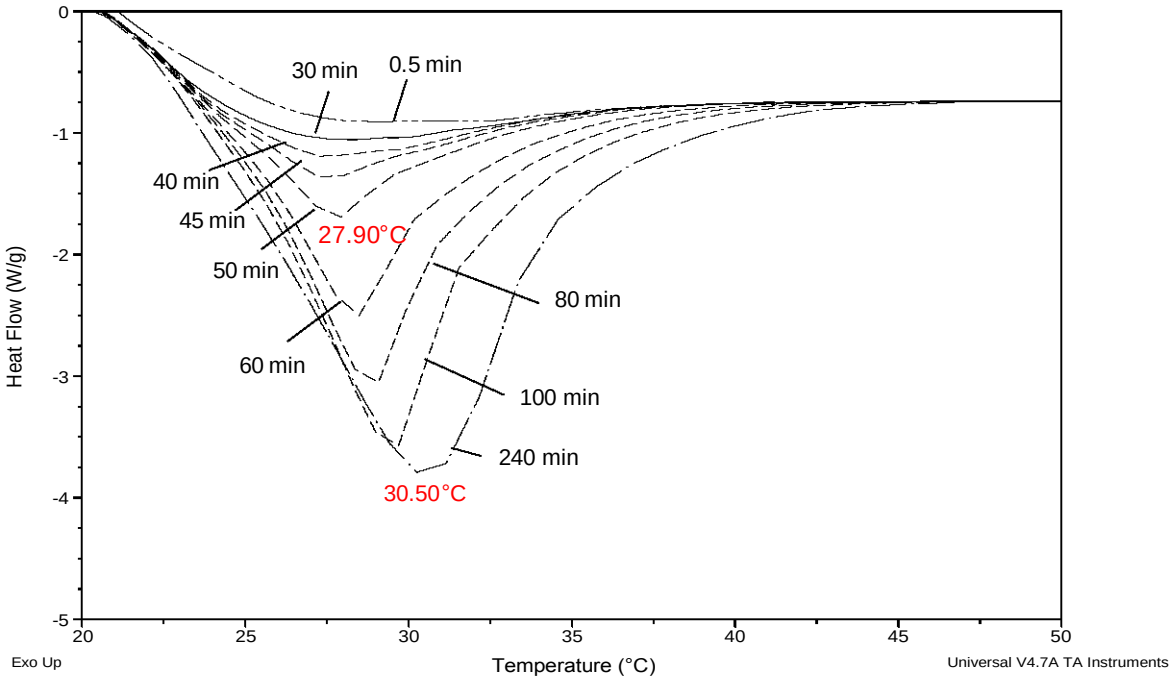
Annex IV

Annex IV-A Overlay of stop and return melting curves of (a) 1.25% DAG (b) 2.5% DAG and (c) 5% DAG at 20°C Isothermal crystallization times and some peak maxima are indicated



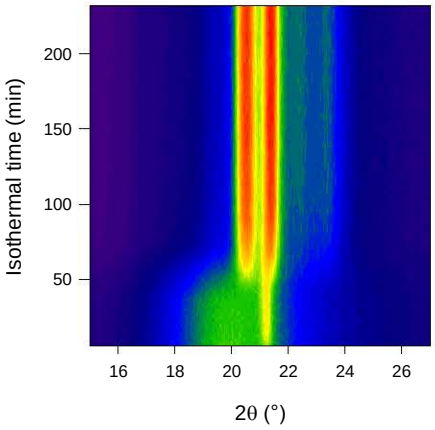


(c)

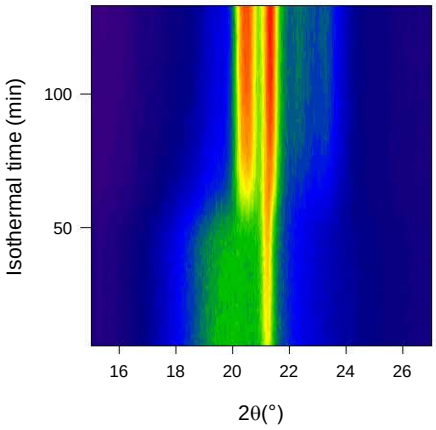

0
-1
-2
-3
-4
-5
20
25
30
35
40
45
50
Heat Flow (W/g)
Temperature (°C)
Exo Up
Universal V4.7A TA Instruments
30 min
0.5 min
40 min
45 min
27.90°C
50 min
80 min
60 min
100 min
240 min
30.50°C

Annex IV-B WAXD patterns of (a) CB, (b) 1.25% DAG, (c) 2.5% DAG, (d) 5% DAG, (e) 10% DAG crystallized at 20°C

(a)



(b)



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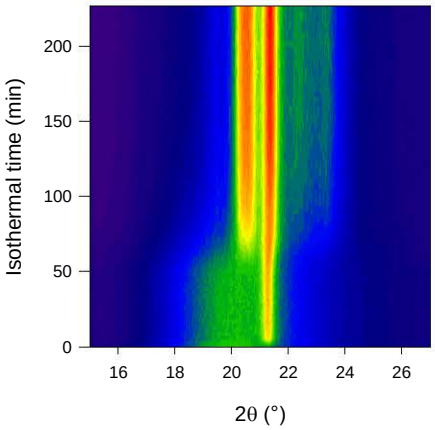
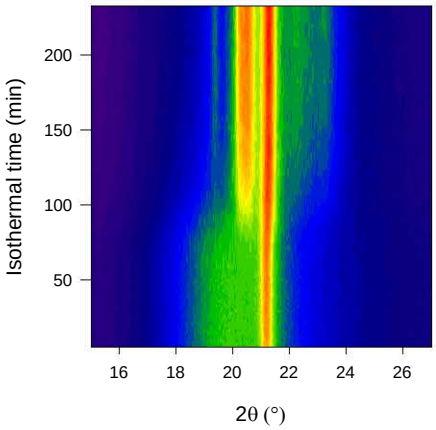


(d)



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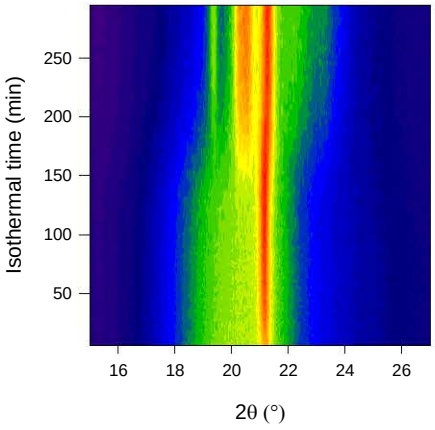

List of abbreviations

CB	Cocoa butter
CM	Cocoa mass
DAG	Diacylglycerol(s)
DSC	Differential scanning calorimetry
FA	Fatty acid
FFA	Free fatty acid
L	Linoleic acid
O	Oleic acid
P	Palmitic acid
PLM	Polarized light microscopy
pNMR	Pulsed nuclear magnetic resonance
POP	1,3-dipalmitoyl-2-oleoyl-glycerol
POSt	Palmitoyl-stearoyl-2-oleoyl-glycerol
S	Saturated fatty acid
St	Stearic acid
TAG	Triacylglycerol(s)
U	Unsaturated fatty acid

Curriculum Vitae

N A T H A L I E D E C L E R C Q

T E L . + 3 2 9 2 2 2 7 8 2 2 • C E L L P H O N E + 3 2 4 7 4 2 1 8 9 1 4

E - M A I L N . D E C L E R C Q @ U G E N T . B E

EDUCATION

1993 – 1999 Science-Mathematics, Instituut Zusters Onbevlekte Ontvangenis, Oosterzele

1999 – 2004 Ghent University, Faculty Bioscience engineering
Master of Bioscience Engineering: Chemistry

- Master thesis: Development of a colour predicting model for imitation chocolate (Laboratory of Food Technology and Engineering)
- Promotor: Prof. dr. ir. Koen Dewettinck

2004 – 2006 Ghent University, Faculty of Psychology and educational sciences:
Master of educational sciences in the applied biological sciences

PROFESSIONAL ACTIVITIES

2004 – 2007 PhD Student Laboratory of Food Technology and Engineering, (FTE). PhD research: Changing the functionality of cocoa butter, in collaboration with Barry Callebaut, Promotor Prof. dr. ir. Koen Dewettinck

2007 – 2011 Assistant at the Laboratory of Food Technology and Engineering, (FTE) PhD research: Changing the functionality of cocoa butter, Promotor Prof. dr. ir. Koen Dewettinck

2005 – 2011 Tutor of 10 masterstudents, 2 bachelor projects

2007 – 2011 Responsible for the practical exercises of Food Processing and Food Technology

SCIENTIFIC ACTIVITIES

(a1)

Depypere, F., De Clercq N., Segers, M., Lewille, B., Dewettinck, K. (2008). Triacylglycerol migration and bloom in filled chocolates: Effects of low temperature storage. European Journal of Lipid Science and Technology, 111, 280-289.

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De Clercq, N., Moens K., Depypere, F., Vila Ayala, J., Calliauw, G., De Greyt, W., Dewettinck K. Influence of cocoa butter refining on the quality of milk chocolate. Journal of Food Engineering. Submitted.

(a2)

De Clercq, N., Foubert, I., Dewettinck, K. (2008). Separation and analysis of acylglycerols by chromatographic methods. Lipid Technology, 20, 232-234.

(a4)

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(c3)

De Clercq, N., Foubert, I., Dewettinck, K. Separation and analysis of acylglycerols by chromatographic methods. 98th AOCS Annual Meeting & Expo, Quebec, Canada, 13-16 May 2007. Oral presentation.

De Clercq, N., Foubert, I., Dewettinck, K. Separation and analysis of acylglycerols by chromatographic methods. International convention center, Ghent, Belgium, 17-21 June 2007. Poster Presentation.

De Clercq, N., Foubert, I., Dewettinck, K. New innovative fats and oils with a healthy fatty acid profile. European Congress of Chemical Engineering-6 Copenhagen, Denmark, 16-20 September 2007. Oral presentation.

De Clercq, N., Vereecken, J., Dewettinck, K. Melting behaviour of some nutritionally interesting oils blended with palm oil and its fractions. 99th AOCS Annual Meeting & Expo, Seattle, USA, 18-21 May 2008. Oral presentation.

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Delbaere, C., Depypere, F., De Clercq, N., De Pelsmaeker, S., Januszewska, R., Gellynck X., Dewettinck, K. Effects of low- and high-temperature storage on oil migration and fat bloom development in filled chocolates. Schoko-teknik, Köln, Germany, 7-9 December 2010. Poster presentation.

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Een terugblik

Als er één ding is waar ik in geloof dan is het “het lot”.
Na de boeiende lessen van Prof Huyghebaert was ik wel iets voor voeding gaan voelen. Daarom beslisten 2 meisjes begin 2003 om bij Prof. Dewettinck langs te gaan voor wat informatie over de thesisonderwerpen. Met bange hartjes klopten we aan. We werden begroet door een wat norse man die ons niet eens binnenvroeg. Dat beloofde! Na dat gesprek stond het onderzoek over croissants op nummer 1. Groot was dan ook mijn teleurstelling toen ik enkele maanden later vernam dat ik maar mijn derde keus kreeg, iets met kleur op chocolade i.s.m. Barry Callebaut. Gelukkig was ik een notoir liefhebber van de olifant dus ik legde me neer bij mijn lot.
Wat als ik het onderzoek op de croissants had gehad? Dan was Filip me tijdens het chocolade maken (wat toen nog op redelijk primitieve wijze gebeurde ergens in een verborgen lokaaltje) misschien nooit durven komen vragen of ik eens met hem wou uitgaan.
Ik zou waarschijnlijk in juni 2004 ook nooit een telefoontje gekregen hebben van Imogen met de vraag of ik wou doctoreren op een project met Barry Callebaut. Het kwam als een geschenk uit de hemel, ik heb dan ook geen moment getwijfeld want ondertussen had ik al de kriebels voor onderzoek te pakken.
En zo komt het dus dat het lot mij op de plaats waar het allemaal begon, lokaal A0.28, laat schrijven aan de laatste pagina’s van mijn doctoraat. De cirkel is rond.

Het heeft wel wat voeten in de aarde gehad vooraleer dit werk op papier is geraakt. Het gerucht bereikte me zelfs dat Nathalie nooit haar doctoraat zou halen. Haha, dat moet je natuurlijk niet tegen mij zeggen. Het was juist een extra stimulans. Mijn persoonlijk motto is dan ook: Geef nooit op! Als ik er nu op terugkijk, had ik dit werk al wat eerder kunnen neerleggen. Ik had misschien ook af en toe wat meer aan mijzelf moeten denken maar zo zit ik niet in elkaar. Het lot gooide er dan ook nog eens een prachtig, fantastisch kindje tegenaan en opeens werd alles zo relatief. En zo zijn voor je het weet 7 jaar voorbij gevlogen. De laatste maanden van die periode waren toch wel redelijk zwaar. Ik heb mij een drietal maanden in totale eenzaamheid moeten opsluiten in mijn schrijvershol. Bij een gebrek aan inspiratie, wanneer Sigmaplot voor de zoveelste keer uitviel of gewoon niet opende, wanneer de pagina’s moeilijk gevuld raakten was de redding gelukkig altijd nabij: bovenste schuifje rechts: mijn chocoladevoorraad! Wat ook hielp was de muziek eens vollen bak, luid meezingend (bureaugenootjes, wees dus blij dat ik thuis zat) *What if I found the guiding beat? And found the card to stop the beating heart.* Zalig nummer! Mijn verplichte retraite heeft uiteindelijk wel iets opgeleverd waar ik toch bescheiden trots kan op zijn. Het

kan natuurlijk altijd beter maar ik heb er mij ondertussen bij neergelegd dat in onderzoek perfectie nastreven hetzelfde is als mij vis laten eten, onmogelijk dus.

Van al dat mijmeren ben ik eigenlijk aan het afdwalen van het eigenlijke doel van deze laatste pagina's in mijn boekje: het bedanken van een aantal mensen.

Eerst en vooral, de norse man bleek uiteindelijk nog wel mee te vallen ☺. Beste promotor, Koen, ik ben je echt zeer dankbaar voor de kansen die ik de afgelopen jaren gekregen heb. Ik heb mijn weg voor een groot deel zelf mogen uitstippelen en dat kon alleen maar omdat je een enorm vertrouwen in mij had. Ik hoop dat ik dit vertrouwen vandaag heb waargemaakt. Ik kijk uit naar onze verdere samenwerking om verder te bouwen aan FTE en het Cacaolab verhaal!

Het onderzoek op cacaoboter was niet mogelijk geweest zonder de financiële steun van Barry Callebaut. Het project werd mee op de rails gezet door Dirk Poelman en Herwig Bernaert. Daarna nam Karen Casier het van hen over. Bedankt voor de tijd en de middelen die jullie geïnvesteerd hebben in dit onderzoek.

Het eerste deel van het onderzoek is gebaseerd op de resultaten van masterthesis onderzoek in samenwerking met Desmet Ballestra Group. Bedankt José, Wim en Gijs voor de constructieve samenwerking.

De examen en leescommissie, Prof. dr. ir. Chris Stevens, Prof dr. ir. Paul Van Der Meeren, Prof. dr. ir. Frédéric Depypere, Prof. em. dr. ir. Roland Verhé, Prof. dr. ir. Sabine Danthine, Dr. Kevin Smith, Dhr Alex Landuyt wens ik te bedanken, remercier, to thank voor de tijd die ze vrijmaakten om het proefschrift te beoordelen. Jullie bemerkingen en suggesties waren zeer waardevol voor het finaliseren van dit proefschrift.

En nu de collega's, met wie moet ik beginnen? Het is een hele waslijst! Ik zou eerst algemeen willen zeggen dat de afgelopen jaren binnen FTE er waren met veel arbeidsvreugde en dat vooral dankzij de toffe collega's. Naast het serieuze werk kon er gelukkig ook eens gelachen worden. Vooral de koffiepauzes, in mijn geval de fruitpauzes, waren er om even alles aan de kant te schuiven, wat te roddelen of om vooral op vrijdag vettige praat te verkopen. Dat dagelijks kwartiertje (of heel soms iets langer) zijn zo belangrijk voor de sfeer binnen de groep. Ik hoop dat we in de toekomst zonder koffieruimte toch nog ergens een plekje vinden om dat zo belangrijke groepsgevoel te behouden.

Ik heb voor de verwezenlijking van dit doctoraat heel wat hulp gekregen in het labo. Ik vergeet Monique niet, die zich in de beginjaren over mij ontfermd heeft. Griet, die van de embryo's in de dierengeneeskunde in de chocolade werd gegooid. Griet, ik denk dat je stilaan een echte chocolatier geworden bent. Bedankt voor de vele keren dat ik op jou heb kunnen rekenen! Speciale vermelding gaat toch uit naar BENNY! De rots van ons labo! Mijn sproeidroogmaatje, mijn maatje om eens een klapke mee te doen. Je staat echt waar ALTIJD voor iedereen klaar! Chapeau!! En natuurlijk, Benny's grappen en grollen zijn ongenaakbaar. Het begint met een kleine gniffeltje gevolgd door een imitatie van een tomaat. Hilarisch!!!

Ik ben in mijn hele carrière nooit van bureau verhuist maar ik heb de samenstelling vaak zien wijzingen. Het is begonnen met Imogen, mr. Temptation Dirk en babbelwater Veerle. Imogen, ik heb heel wat van jou geleerd. In dit doctoraat zitten er ook nog heel wat van jouw ideeën, ik ben dat nog niet vergeten. Je was zo'n beetje mijn leermeester de eerste jaren. Bedankt hiervoor. Toen werd mijn bureau langzamerhand een mannenbastion met de komst van Jeroen, Piet en Maarten. Merci Jeroen, voor het doorgeven van de spelletjesmicrobe. Dankzij jou hebben we ondertussen al een redelijk grote collectie. Ik hoop dat we in de toekomst nog veel avonden mogen doorbrengen aan de spelletjestafel! De minst opvallende is Maarten. Ik heb je de afgelopen jaren toch zien openbloeien. Geloof vooral in jezelf. Sinds vorig jaar hebben twee heel toffe madammen, Kim en Stefanie hun intrek genomen. Ik heb de laatste maanden heel hard op jullie kunnen rekenen en dus ik sta zeker en vast bij jullie in het krijt! Kom dat maar op tijd innen! Ik sta altijd klaar om te helpen waar het kan!

Dan is er ook nog "den anderen bureau", ook heel toffe mensen: glorious bastard en dé zetmeelspecialist Bart, liefhebster van geestrijke vloeistoffen Claudia en (ex)-collega assistentje Eveline. Eveline, ik vond het super dat we steeds zo goed hebben kunnen samenwerken. We begrepen elkaar meestal zonder al te veel woorden. Veel succes op 11/10 en +/- 24/03!
Dan zijn er nog twee mannen die ik zeker niet mag vergeten vermelden omdat ik altijd op hen kan rekenen voor naleeswerk, goede raad,…: Frédéric en grapjurk Davy. Jullie verdienen ook een heel oprechte dikke merci!
Bij wie het altijd gezellig druk is, is bij de mama's van de buitenlandse studenten Katleen en Ruth vandaar dat ik er altijd graag eens binnenwandel. Katleen, ik kijk al uit naar onze KOI!
Dan zijn er ook nog de eeuwig jonge Paul, Beatrijs, Corine, Marleen, mijn 2 superthesisstudentjes Liesbeth en Elien (en ik hoop nu dat ik niemand vergeten ben): elk hebben jullie op jullie manier bijgedragen tot mijn werkstuk.

The last years, a lot of new students arrived from all over the world. Good luck with your PhD research the coming years! You can always count on me!
Het laatste jaar is er niet echt veel tijd geweest voor ontspanning. Mijn vriendinnetje van het eerste uur Boerekot Karin, Annelies en Selin: we zitten momenteel nog maar net aan mijn verjaardag voor onze Ladies nights dus we zullen nog een serieuze inhaalbeweging moeten inzetten de komende maanden want binnenkort moet ik alweer een kaarsje uitblazen!
Voor mijn andere vriendjes en vriendinnetjes: ik moet tot mijn grote scha en schande toegeven dat ik de laatste maanden en voor sommige misschien wel jaren niet echt veel tijd gehad heb. Ik hoop dat binnenkort de Lustige Droppers nog eens van stal komen. Gelukkig staat er dankzij Sabrina, who else, voor volgend jaar toch al een weekendje gepland!

Op wie ik altijd kan rekenen voor om het even wat, dat zijn mijn mama en papa. Nu ik zelf mama geworden ben, besef ik wat het is om ouder te zijn. Het is meer dan alleen maar je kind graag zien, het is er alles voor over hebben. Mama, ik ben je zo dankbaar dat je op mij ingepraat hebt toen ik tijdens de examens van eerste kan niet meer verder wou studeren. Ik had hier vandaag niet gestaan. Ik hoop dat jullie trots zijn op wat ik bereikt heb want het is ook een deel jullie verwezenlijking. Zonder jullie onvoorwaardelijke steun was dit nooit gelukt. Dat geldt natuurlijk ook voor mijn zusje. Je hebt trouwens nog altijd een etentje tegoed voor mijn thesis. Bij deze staat het nog eens op papier. Ik ben het nog niet vergeten ☺. We kunnen in ruil natuurlijk ook altijd eens gaan shoppen met Paulientje!

Peter, schoonouders Marleen en Freddy, Evelien en Gino, Bart: bedankt voor jullie steun!

Dan blijven er nog dé twee mannen uit mijn leven over: Filip en Lander. Lander, mijn lief, “klein” bolleke, ik heb je de laatste maanden vaak met veel pijn in het hart moeten missen. Je hebt er waarschijnlijk niet veel van gemerkt want je werd altijd schitterend opgevangen door papa, oma, oma Erpe. Je bent ondertussen een grote kapoen en (waarschijnlijk met de mamamelk meegekregen) een echte chocoladeliefhebber! Opvolging verzekerd!
Mijn Filipke, we hebben een jaar achter de rug dat niet te snel moet terugkeren. Allebei een doctoraat: jij geen tijd voor mij en ik geen tijd voor jou. Het was hier vaak een beetje hectisch. Je hebt me gesteund, me de juiste weg getoond toen ik door de bomen het bos niet meer zag, XRD data op een sneltempo verwerkt, alles grondig en kritisch nagelezen, het huishouden gedaan, Spam, onze vis ververst (voor de insiders, Spam zwemt nog steeds gelukkig rond in zijn aquarium), … M.a.w. je bent mijn SUPERman! *As science struggles on to try to explain, oxytoxins flowing ever into my brain.*

Hier zet ik mijn finale punt *Message End.* Nathalie (*@NathieDC*)